EXPERIMENTAL CELL RESEARCH

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H. BAUER · T. CASPERSSON · E. FAURÉ-FREMIET
H. B. FELL · G. MONTALENTI · J. T. RANDALL · J. RUNNSTRÖM
F. O. SCHMITT · P. WEISS · R. W. G. WYCKOFF

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EDITORS:

H. BAUER, Max Planck Institut f. Meeresbiologie, Wilhelmshaven 23, Germany
T. CASPERSSON, Department of Cell Research, Karolinska Inst., Stockholm 60, Sweden
E. FAURÉ-FREMIET, Collège de France, Paris V:e, France
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J. T. RANDALL, Wheatstone Physics Laboratory, King's College, London W. C. 2, England
J. RUNNSTRÖM, Wenner-Gren Institute for Experimental Biology, Stockholm Va., Sweden
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MITOCHONDRIA IN *ALLOMYCES* UNDER EXPERIMENTAL CONDITIONS

D. RITCHIE and PATRICIA HAZELTINE

Department of Botany, Barnard College, Columbia University, New York, N.Y., U.S.A.

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THE mitochondria of various tissues from various organisms are not identical. That there are differences in the physical organization is shown by the fact that the mitochondria of guinea-pig liver can be isolated in 0.88 M sucrose (19), but those of amphibian eggs are destroyed in that medium (32). Variation in chemical composition has been shown by Hogeboom and Schneider (18), who demonstrated by means of the analytical centrifuge that there are, in mitochondria of normal cells, protein fractions which are different from those in hepatoma cells. Because such differences are known to exist, some biologists have searched for a cytoplasm lacking the special activities that can be expected in specialized cells.

A desire for a source of undifferentiated cells was responsible for the choice of leukemic cells by Claude (8). He sought undifferentiated cells whose cytoplasm was not specialized and which ought not therefore exhibit any mitochondrial idiosyncrasies. For the same reason, Strangeways and Canti (37) interested themselves in tissue culture cells of the embryonic chick. Until more is known of the structure and function of cytoplasmic particles, it seems desirable to study them in the least specialized cells obtainable.

It is difficult to imagine less specialized cells than those of vegetative fungus mycelium. Here is a minimum of complication which, in more highly evolved tissues, might confuse the picture. The cell wall, relatively free from structural peculiarity, is a transparent cylinder. The nuclei are never so numerous as to obscure the cytoplasm; and at least in old cells, the fat droplets, which are the only other particles in the hyphae, are too few to cause difficulty in observation. Not only are such cells structurally undifferentiated, but in their ability to serve in conduction, general metabolism, or reproduction as occasion demands, they have a potential functional omnipotence.

The necessity of mounting living tissue in liquid for microscopic observation makes it desirable to use an organism whose normal habitat is in water.

Hyphae of an aquatic Phycomcete, in being spread in a layer one cell thick on a slide, are not subjected to the shock that most tissues receive upon being so treated, and, once mounted, are in a normal environment. The desirability of providing a defined and reproducible environment having been discussed at length by Carrel (7), no more need be added at this time.

Besides naturally occurring in an aquatic habitat, the undifferentiated, transparent hyphae of the genus *Allomyces* have an outstanding characteristic which makes them especially useful for cytoplasmic investigation. This is that the mitochondria in *Allomyces* are so large, numerous, and conspicuous that even by means of an ordinary light microscope they can be seen without special treatment, and may be subjected directly to experimental conditions. It is not necessary to kill the organism, or section or macerate its tissues in order to get at the desired parts.

With phase contrast optics, the mitochondria of *Allomyces* are clearly visible and fascinating objects, seeming blue-black in color. Under optimum conditions they resemble an aggregation of vigorous earthworms. In addition to the twisting, turning, and progressive movement described by Strangeways and Canti (37), mitochondria are capable of other movements which are even more like those of worms. A mitochondrion in *Allomyces* can shorten and thicken, or lengthen and become slender; or a lump may pass along its length like a rat being swallowed by a snake. Mitochondria can be seen to swell, coil, branch, fragment, coalesce, put out pseudopods, even to get tied in knots. They can change their form until they resemble bubbles, strings of beads, dumbbells, lemons, or snow shoes. And they can disappear either by fading slowly from sight or by breaking into ever smaller fragments until they pass below the limit of visibility.

For observation, hyphae were severed from the substrate (hemp seed) and mounted in distilled water. Since the septa in *Allomyces* are incomplete, the mycelium is physiologically coenocytic, and it might therefore seem that cutting the base of a plant away from its substrate might disturb its metabolism. Judging by general appearance, cytoplasmic streaming, and reproductive ability, the disturbance is not serious. The wound closes over within a few minutes. Although we have assumed for practical reasons that the severed hyphae were normal, we have not forgotten that we are not working with intact plants, nor that we may have introduced changes of which we are not aware.

The organisms used in these experiments were four species of *Allomyces: A. anomalus, A. arbuscula, A. javanicus,* and *A. moniliformis.* They are the same strains that were used in a previous study (33).

Osmium tetroxide

The experiments of Belar (1) and Strangeways and Canti (37) have shown that OsO_4 causes no visible distortion of cells. This is as true for *Allomyces* as it is for grasshoppers and chick embryos. Upon being drawn under the cover glass, a 1 per cent solution OsO_4 causes a shudder to pass along the cytoplasm of a hypha, but the appearance of the cells and of all their components remains after treatment the same as it was before. When it became apparent that live mitochondria (Figs. 1, 4) were usually too active to photograph satisfactorily because of their motion, it was decided that cells fixed in OsO_4, being normal in shape and having the virtue of immobility, would be used as a standard of comparison (Figs. 2, 5, 13). In experiments on the mitochondria, change or lack of change of the cellular components was determined by direct observation, but in presenting the results here, most of the so-called normal mitochondria are in fact dead, their activity having been stopped by fixation. Dead though they are, however, their shapes, sizes, and refractivity are the same as they were in life, and it is not possible to determine, simply by inspecting a photograph, whether a mitochondrion is alive or has been killed in OsO_4. Brownian movement of the smallest cytoplasmic particles continues after fixation. The mitochondria are hardened, with the result that they are better able to withstand mechanical shock than they were when alive. Slight pressure on the cover glass does not cause the disintegration of fixed mitochondria.

Sodium chloride

In a graded series of solutions, the most concentrated one which causes no visible change in the size of the protoplast is in these experiments considered isotonic. In *A. javanicus*, a 0.21–0.24 M NaCl solution is isotonic. When hyphae are mounted in such a medium, the mitochondria are as sensitive to mechanical shock as when mounted in water, pressure on the cover glass causing immediate fragmentation of filamentous and short rod mitochondria. The diameter of the resulting granules is about equal to the thickness of intact mitochondrial filaments. In this, *Allomyces* seems to differ from mammalian tissue (9), which will yield up its mitochondria, at least in granular form, in isotonic saline. It should be noted that "tonicity" as used in the present paper does not mean what it does when employed by animal physiologists.

It has not been possible to watch a mitochondrion escape from a ruptured hypha and exist intact in the surrounding medium. Such other cell components as nuclei and fat droplets do this, but mitochondria never. In fact, to the best of our knowledge, no one has observed the liberation of a single intact mitochondrion from its position in a living cell out into the surrounding medium, although Warburg (38) long ago came close to doing that. It seems desirable that such an observation be made, even though the particulate matter which can be isolated by differential centrifugation seems to be mitochondrial.

In 0.7 M NaCl, which is of course hypertonic, the cells undergo sudden and severe plasmolysis, which is followed by gradual recovery. Mitochondria are at first not affected, but within a few seconds, the churning cytoplasm fragments them into small spheres (Fig. 10). After cells recover from plasmolysis, mitochondria swell, becoming ever less refractive. They become ameboid, put out pseudopods, fragment, coalesce, move about (Fig. 7). As they become larger and paler (Fig. 11), they can be seen to contain a number of light spherical "vacuoles" of various sizes (Figs. 3, 12). We know nothing of the contents of the light spots in the mitochondria, and have referred to them as "vacuoles" because of their superficial resemblance to true vacuoles.

No acceptable explanation for this behavior of the mitochondria in hypertonic saline has been discovered. Opie's (29) experiments with liver in distilled water indicate that what he calls cytochondria function as osmotic systems, and he believes that the cytochondrial outer layer is a selectively permeable membrane. This is in support of the similar conclusion of Claude (10). Since no undoubtedly intact mitochondria have been freed from the hyphae of *Allomyces*, it is not known what their action in distilled water would be. Their swelling within hyphae in strong saline solution might be caused by action of the solute on mitochondrial proteins, but speculation is idle.

Sucrose solutions

The solution which Hogeboom, Schneider, and Pallade (19) found successful in isolating mitochondria from mammalian liver tissue was 0.88 M sucrose. When distilled water, in which the hyphae of *Allomyces* were mounted, was replaced by such a medium, plasmolysis was immediate and severe. Within a few seconds after this happened, the filamentous mitochondria broke up into minute spherical fragments, averaging about 1 micron in diameter, which were more stable than the fragments resulting from treatment with NaCl. They were not seen to swell, nor change shape, nor flow together. Sucrose, then, seems to be less damaging than NaCl to mitochondria.

Of course, the fact that fragmentation occurs when cells are immersed in sucrose solution is indication of some damage, and the tiny mitochondrial fragments may be, in spite of some similarity to the original mitochondria, very different from them. That the difference is worth consideration is apparent from experiments which show how much of an organism's activity is determined by its organized completeness. Bodine (5), in comparing oxygen uptake in intact grasshopper embryos with that of tissue homogenates, found that 65 per cent of the total oxygen uptake was due to what he terms "physical structure or intactness."

Potassium cyanide

There is a large and ever-increasing list of respiratory enzymes which are thought to be partially or wholly associated with mitochondria. It would be interesting, therefore, to know what effects, if any, a respiratory inhibitor would have on the morphology of these organelles. In the present study, we followed essentially the procedure used by Shoup and Wolf (35), who studied the effect of KCN on the oxygen uptake of four species of *Allomyces.* They showed that a maximal concentration of KCN, in the presence of a respirable substrate, inhibited about 80–90 per cent of the normal oxygen uptake.

We used *A. javanicus* for this series of experiments. Hyphae from three-day hemp seed cultures were mounted in distilled water and checked for normal appearance. While a cell, chosen for the visibility of its mitochondria, was kept under observation, water was drawn off from one side of the cover glass and was replaced by a 10^{-2} M solution of glutamic acid at pH 6.8. This action produced no visible effect on any portion of the cells, the mitochondria remaining active in their customary fashion. After about half an hour, the glutamic acid was replaced by a mixture of glutamic acid and KCN, in which the concentration of the KCN was 10^{-2} M (pH 7.0). Within five minutes, all active cytoplasmic movement ceased, although there was no apparent physical damage to any of the cell parts (Figs. 6, 9). The mitochondria retained their original size, shape, and refractivity, but remained motionless. Ryland (34) reported that KCN caused coagulation, fragmentation, or coalescence of mitochondria in onion and narcissus root tips. In killed, sectioned, and stained tissues, she could find no filamentous mitochondria after treatment.

Staining of mitochondria of tissue culture cells with methylene blue is inhibited by KCN (23). Similarly, the mitochondria of *Allomyces* cannot be stained by Janus green after cyanide treatment. Hyphae mounted in 0.001

per cent Janus green at pH 7.0 will sometimes show blue mitochondria, but we could never observe staining of KCN-treated cells. If Marston's (26) explanation of the action of Janus green staining is correct, this would indicate that in addition to blocking respiratory enzymes, cyanide also affects the action of proteolytic enzymes, and gives little-needed support to the statement by Green (16) that the mitochondrial enzyme complex "can be treated as if it were a single entity." In the absence of cyanide, 30 minutes to an hour are required for accumulation of dye. It is interesting that subsequently the protoplasm coagulates, the fat droplets coalesce, the mitochondria disintegrate, and the whole hypha turns blue. In the concentration employed, the "vital" dye is more drastic in its action than cyanide.

Temperature effects

The effects of high temperatures on mitochondria have been briefly reviewed by Belehradek (2). In *Allomyces*, mitochondria were observed in distilled water on a heated stage, while the temperature was raised in five-degree steps, at ten minute intervals. When the temperature reaches 50° C., the mitochondria become less refractive and so slender as to be almost invisible, but as the temperature continues to rise, no further change occurs until at 68° C. the mitochondria sometimes become moniliform. These results agree in general with those of Policard and Mangenot (30) and Famin (14). At 75° C., the mitochondria break up into beads which become spheres with transparent centers and then separate from one another, finally disappearing. No independent movement was recorded above 65° C., but hyphae held at 69° C. for ten minutes possessed mitochondria which, upon being returned to room temperature, seemed normal and have been seen to move.

Lowering of the temperature produced no morphological effect on the mitochondria, but did in most trials reduce the rate of protoplasmic streaming. A temperature of 8° C. is usually low enough to slow streaming and cause the mitochondria to lose their orientation.

Pollister (31) pointed out that mitochondria in the liver cells of *Amphiuma* were oriented in lines which were indicative of the diffusion gradients within the cell. Similar orientation has been since observed in other cells and other organisms. Lewis (cited in Bourne, p. 233) described round trips of mitochondria from the nucleus to the periphery of the cell. The explanation for these facts could lie in the occurrence, easily seen in *Allomyces*, of cytoplasmic currents which cause the mitochondria to line up.

In the essentially coenocytic hyphae of *Allomyces*, the diffusion gradients of oxygen and soluble foods into and of waste products out of the cell must be perpendicular to the long axis of the hypha. The main currents of protoplasmic streaming are along the axis. As long as streaming is strong, the mitochondria are, sometimes with striking uniformity, lined up parallel to the hyphal axis. When streaming is slow the uniformity is less noticeable, and if it ceases, the mitochondria can be seen to lie in all directions (Fig. 6). The correlation between protoplasmic streaming and orientation of the mitochondria in *Allomyces* is complete and readily observable, and it is likely that mitochondria in general are oriented by the direction of streaming of the cytoplasm which may not be the same as the direction of diffusion.

Reduction of triphenyltetrazolium chloride

In order to find the site of reduction of tetrazolium salt to the red formazan, we planted hyphal slips on hemp seed in Petri dishes containing 20 ml. distilled water plus the reagent in the following concentrations: 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 and 1.0 per cent. All solutions with as great a concentration as 0.01 per cent were lethal, but in a 0.005 per cent solution, colored granules could be seen after 48 hours (Fig. 8). The granules, assumed to be mitochondria, were small spheres. Though they were obviously neither fat droplets nor nucleoli, they were never filamentous as typical *Allomyces* mitochondria are, and they exhibited no independent movement. In hyphae which were killed by the salt, the entire cellular content stained intensely, particularly in the hyphal tips where the cytoplasm is most active. Mitochondria are commonly most easily observed below the tips, where other cytoplasmic particles are less numerous. When the plants were kept under observation during the time of action of the tetrazolium, the first and strongest reaction was to be seen in the fat droplets. That these inclusions are indeed fat droplets has been determined (33).

Size of mitochondria

"The diameter of mitochondria in individual cells is extraordinarily constant, and though they may increase in size they never do so by increasing their girth, but by an increase of length." (6, p. 233.) This statement is based on observations of mitochondria in special cells, mainly in animal tissues such as kidney, liver, various epithelia, and tumors. In *Allomyces*, the differences in length of the mitochondria are great, the longest under normal conditions being possibly 50 times as long as the shortest. The longest measured mitochondrion was about 25 micra long, but some active ophidian ones were estimated to be twice that. The variation in thickness is certainly less, affording a ratio of about 10 to 1.

Since the thickness of the mitochondria is an important factor in their visibility, a preliminary portion of the present study was concerned with finding what conditions were conducive to the most readily observable mitochondria, and it was noted that in general they were easiest to see when the cultures were incubated at 30° C. rather than at lower temperatures. Repeated trials with higher temperatures showed that at about 50° C. they became much more slender, and most of them became so attenuated at that temperature that it required persistent watching to make certain they were still present. They also changed their refractivity, so that instead of appearing dark with phase contrast optics they appeared pale yellow, and this color change in addition to the extreme thinness made them all but invisible. Only a crude guess as to their actual thickness could be made at such a time, but by using an eyepiece micrometer it was estimated that they approached 0.2 micra, which is theoretically too thin to be visible with white light.

In some cultures, in reaction to forces unknown to us, the mitochondria became

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(Photographs were made with a Bausch & Lomb microscope with dark contrast phase optics (except Fig. 8), and a Leitz Makam camera. Negatives had an original magnification of 905 ×, and were enlarged to make final prints. Film was Ansco Isopan, developed in developer D-8 to increase contrast. Prints were made on high speed Varigam paper with No. 10 filter, and developed in D-8. Exposures varied from one-half second with carbon arc illumination, to 3 seconds with a No. 1 Photoflood in the microscope lamp. Figures reduced about one tenth in reproduction.)

Fig. 1. Mitochondria, alive, unstained, active. The blurred outlines are the result of movement, which was fast enough to cause fuzziness. *A. javanicus.* × 2260.

Fig. 2. Typical mitochondria, supposedly normal. Movement stopped with OsO_4. *A. moniliformis.* × 1675.

Fig. 3. Plasmolysed cell of *A. javanicus* in hypertonic saline solution, with large, vesiculate mitochondria, ghostly pale. "Vacuoles" are visible in the mitochondria marked by arrows. *A. javanicus.* × 1675.

Fig. 4. One live, active mitochondrion. "Stopped" by short exposure on photographic plate, with resultant loss of contrast. *A. javanicus.* × 1225.

Fig. 5. Surface view of a hypha, with numerous peripherally arranged mitochondria, normal in shape and position, motion stopped with OsO_4. *A. moniliformis.* × 1625.

Fig. 6. Mitochondria of *A. anomalus*, with movement stopped by action of KCN. Instead of being oriented in longitudinal rows, the mitochondria are placed in random positions, but morphologically unaltered. × 1675.

Fig. 7. A single mitochondrion, swollen and lumpy, under influence of hypertonic saline, photographed a few seconds after plasmolysis of the cell. Three degrees of refractivity are visible within the mitochondrion: 1) a dark central granule, surrounded by 2) a paler area, and 3) the ends of the particle, of intermediate intensity. *A. moniliformis.* × 1810.

Fig. 8. Granules in cytoplasm, red with reduced tetrazolium salt. Ordinary light microscope, green filter. Forty-eight hours in 0.005 per cent tetrazolium. *A. arbuscula.* × 2170.

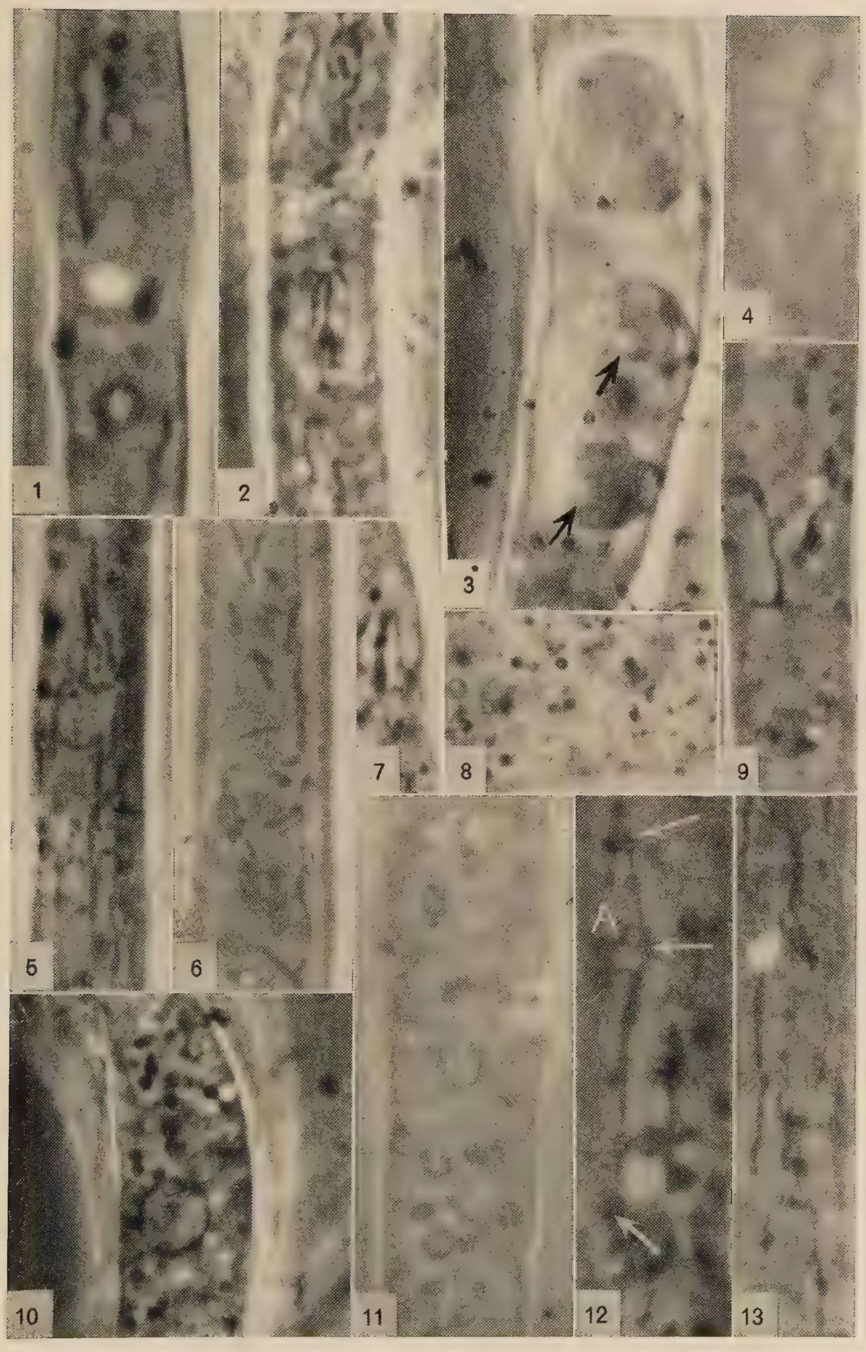
Fig. 9. Cell in KCN solution. One long, normal mitochondrion in center, nucleus with nucleolus at bottom. *A. anomalus.* × 1515.

Fig. 10. Portion of hypha in hypertonic saline, a few seconds later than Fig. 7. Mitochondria spherical, refractive, distinct. Nucleus just below center. *A moniliformis.* × 1990.

Fig. 11. Portion of hypha in hypertonic saline. Spherical mitochondria are much swollen, with low refractivity. *A. javanicus.* × 1500.

Fig. 12. Mitochondria under effect of hypertonic saline, at beginning of vesiculation, with a dumbbell at A, and a long mitochondrion showing blisters at arrow points. A temperature of about 75° C. produces similar structures, but heat-treated mitochondria are too slightly refractive to photograph even by means of phase contrast optics. *A. moniliformis.* × 3075.

Fig. 13. Surface view of a portion of a hypha. Motion stopped with OsO_4, but no stain. No aberrations or distortion apparent. *A. moniliformis.* × 1650.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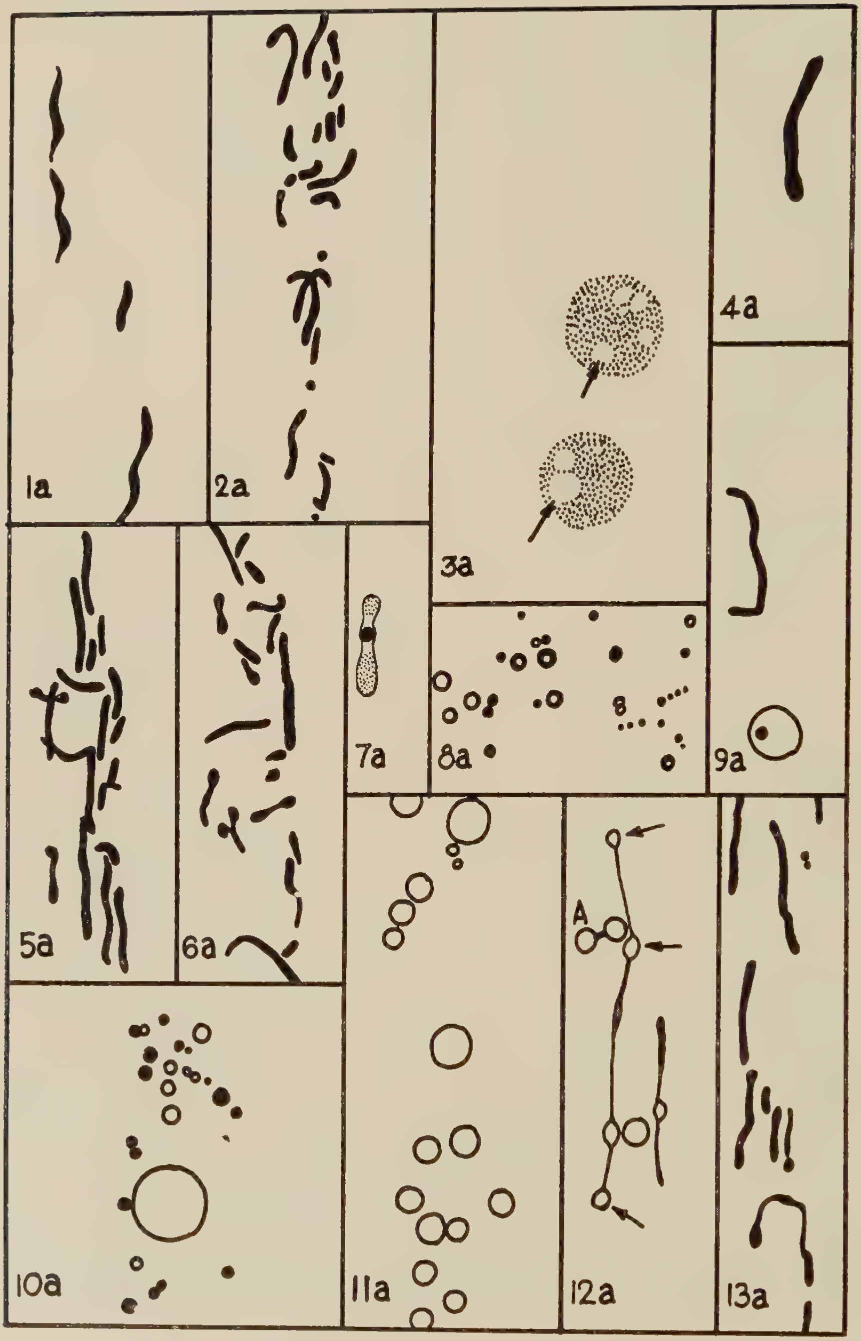
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at times as thin as those in the heated cultures. At their thickest, mitochondria approach 2 micra in thickness, but we have not seen any thicker than that in *Allomyces* except under abnormal conditions, as for instance after replacing a simple aqueous medium with hypertonic saline. The normal variations are great enough to make it clear that inflexibility of girth in mitochondria is not universal.

The mitochondrial sheath

The results of centrifugation on eggs (particularly eggs of marine invertebrates) show that when a sphere is elongated until it is about 3 times its diameter it divides into two pieces (17). The eggs studied have external membranes, but the membranes are not strong enough to hold the body in one piece when a critical length is exceeded. In normal cells, the mitochondria exist in a form so slender that the length may be ten or even twenty times the thickness. This suggests that there is a relatively strong covering on the mitochondria, a covering which when undamaged is capable of maintaining the mitochondrion in a form which would be impossible without a covering envelope. By a kind of plasmoptysis, Zollinger (41) has demonstrated a visible sheath on mitochondria of mouse liver cells in water, and Claude and Fullam (11) have photographed a mitochondrial membrane by means of the electron microscope. The visibility of a membrane (Fig. 11) gives little indication of its strength, but since mitochondria do have visible membranes, and since mitochondria can maintain long slender shapes, it seems that the membranes must be structures of considerable strength.

It is notable that filamentous mitochondria break up into short pieces when subjected to many various treatments. The commonest result is a scattered mass of minute spherical bodies whose diameter is about the same as the thickness of the filament from which they came. This was true in the experiments with tetrazolium chloride and sucrose, and has been noted previously by many observers working on a great variety of cell types. Of greater interest is the fact that the mitochondria do not invariably break up into small spheres, but into short rods, commonly called batonnets in the literature. The batonnets, as shown in numerous published figures and as observed in *Allomyces*, are about the shape of the centrifuged eggs which are about to pull into two pieces. That is, they are about 3 times as long as they are thick, and are as long as the membranes, weakened by some disturbing factor, will permit them to be. In the present studies, heating the mitochondria commonly resulted in the production of a preponderance of the batonnet forms in the range 50°–80° C. These phenomena can be explained if it is assumed that normal mitochondria in a healthy cell possess a relatively tough limiting membrane, but that their strength is reduced by many of the vicissitudes the cell encounters in a biological laboratory. A partial loss of toughness results in mitochondria in the form of short rods, and still greater loss results in minute spheres, the Körnchen of Warburg, or the "large granules" of Hogeboom and other recent investigators. The effect, then, of acid fixing agents or mechanical pressures would be due wholly or in part to the

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Figs. 1a–13a are line drawings taken from the corresponding Figs. 1—13, simplified to clarify the points in question.

changes which they produce in the strength of the mitochondrial cover. That the mitochondria are sensitive to changes in osmotic values has been shown by Opie (29), by Zollinger (41), and by the effects of sodium chloride and sucrose solutions on *Allomyces.*

Nature of mitochondria

It is generally agreed among cytologists that mitochondria are small but visible particles in the cytoplasm of most cells, that they are capable of accumulating Janus green B, that they can move, and that they are more easily disintegrated than most cell components. It is fairly well agreed that they are made up of some lipo-protein complex (3, and others), that in plants they are the precursors of plastids ((21) and many later workers), that they contain ribose nucleic acid ((24) and others), that they are associated in an imperfectly understood way with the formation of hemoglobin in certain erythrocytes (13), that they contain various amounts of several vitamins (4), that they are centers of accumulation of respiratory enzymes ((16) and many others), that they form the middle piece in some spermatozoa (15), and that they are sensitive to osmotic changes (41). They are concerned with glandular secretion in animals (22) and starch accumulation in plants (28), with the presence of melanomas in mice (40), with the elaboration of latex particles in rubber plants (27), with the building of new cell walls in sporogenous fern tissue (25), with digestive enzymes in protozoa (20), and the formation of antibodies in mammals (12). It has been claimed and fairly well substantiated that some mitochondria are autonomous cell components which exist from generation to generation (39). It has also been claimed and fairly well substantiated that they are temporary particles which accumulate sufficient substance to become visible, then may be dispersed (4).

This accumulation of fact and opinion, incomplete enough here, is sufficient to indicate the variety of viewpoints from which cytoplasmic components have been scrutinized, and to show in part why there are so many terms in the literature for cytoplasmic particles. It seems that in the many investigated species of plants and animals, the similarities between various mitochondria are fewer than the differences, and biologists are perhaps not dealing with entities like nuclei, which have many relatively constant structural and functional features, but rather with particles which have some uniformity of shape and the ability to accumulate Janus green. It is not unthinkable that these most conspicuous common characteristics of mitochondria are, if all the facts were known, of relatively minor importance.

SUMMARY

1. The vegetative hyphae of aquatic fungi are described as favorable material for the study of mitochondria in the living state, particularly when observed with phase contrast optics.

2. Immobilization of mitochondria without morphological change was realized by treatment of hyphal cells with 1 per cent solution of OsO_4.

3. When hyphae were mounted in isotonic NaCl, the mitochondria, being as sensitive to mechanical shock as they were when the hyphae were in water,

disintegrated under pressure. In hypertonic saline, cells were at first plasmolysed, but recovered. Mitochondria in plasmolysed cells changed shape, fragmented, became vesiculated, and disappeared after becoming pale and nonrefractive.

4. In cells treated with 0.88 M sucrose, which has been useful in isolating mitochondrial particles from animal tissues, the fungal mitochondria quickly fragmented into spheres about 1 micron in diameter.

5. A solution of 10^{-2} M KCN in 10^{-2} M glutamic acid, at pH 7.0, caused cessation of movement of mitochondria and inhibited staining by Janus green B. No evidence of mitochondrial damage was apparent.

6. Temperatures of 8°–45° C. had no visible effect on mitochondria. At higher temperatures, the results were as follows: 50° C., mitochondria became slender, and almost invisible; at 65° C., all mitochondrial motion ceased; at 75° C., mitochondria broke up into beads which became hollow spheres and disappeared.

7. Mitochondrial orientation is determined more by the direction of protoplasmic streaming than by direction of diffusion.

8. Small granules, never positively identified as normal mitochondria, were "stained" red after 48 hours with triphenyl tetrazolium chloride when the salt was incorporated in the medium at a concentration of 0.005 per cent. Higher concentrations were lethal. The sites at which reduction of the tetrazolium was most active were in the fat droplets.

9. Because of the diversity of behavior and function of the particles referred to as mitochondria, it is suggested that many cytoplasmic particles, even though they do accumulate Janus green, are scarcely comparable to one another.

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THE RATE OF OXYGEN CONSUMPTION IN THE LETHAL HYBRID BETWEEN *TRITON* ♀ AND *SALAMANDRA* ♂[1]

P. S. CHEN

Institute of Zoology and Comparative Anatomy, University of Zürich, Switzerland

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THE cause which leads to the early death of an individual during its embryonic development varies to a great extent. There can be genic or chromosomal changes which result in such violent disturbance in the morphogenetic process that a successful development is no more possible. Or it can be a difficulty of co-operation between the egg and the sperm nuclei which hampers the control of the early development and thus becomes fatal to the life of the individual concerned. The former has been thoroughly investigated from the genetical side under the category of lethal factors, see reviews by Hadorn (40, 41, 42, 43); while the latter has been frequently approached by experiments of merogony, either homospermic, cf. Fankhauser (23–28) or heterospermic, cf. Baltzer (2, 3, 4, 8, 9) and Hadorn (34–39), and hybridization (for references see (5, 6, 7, 10, 19, 20, 44, 45, 46, 51, 52, 59, 60). Whatever the cause may be, our knowledge until now has shown us that, as far as the lethal effect is concerned, there are certain similarities in all these cases; 1) the lethality as a rule comes into effect at a clear and definite period during the development (phase-specificity); 2) the lethal action can be confined only to certain parts of the embryo (cell-specificity); and 3) the affected embryonic tissues or organs can (secondary lethality) or cannot (autonomous or primary lethality) be cured either by transplanting them into another normal individual, or by isolating them from the surrounding lethal environment (7, 11, 41, 53). In addition to the knowledge which we have gained from the morphogenetical point of view, it will be of great interest to know whether physiologically there also exists such a resemblance among both lethal types. Evidently a physiological analysis such as the rate of oxygen consumption in the embryonic development can also give us a deeper insight concerning the ultimate cause of their developmental failure.

During the last spawning season effort was made to follow the respiratory rates of the lethal hybrids between *Triton* and *Salamandra* in the early embryonic stages. The difficulty here lies in the heterogeneous nuclear material in the zygote. With the aid of the knowledge which has been achieved

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by attacking the same problem on different lethal mutants in *Drosophila*, see Chen (22), the cause of which was known to be genic in nature, the present report aims to illustrate the metabolic abnormality which is in striking resemblance in both lethal types.

In the previous study on the oxygen consumption of the three lethal mutants "*ltr*," "*lgl*" and "*lme*" in *Drosophila melanogaster* (22), it has been shown that the respiratory rates of all these lethals were lower than those of the normals as soon as these larvae became morphologically distinguishable. The experimental results demonstrated clearly that a lowering of the metabolic rate corresponded to a defect in the development. The results concerning the partially metamorphosed and the non-metamorphosed homozygous pupae of "*ltr*" deserve our special attention, because there the difference in the rates of oxygen uptake between the two types was detectable even before they became morphologically distinguishable. The observation therefore lended support to the idea that a certain agreement exists between respiration and morphological differentiation.

The first experiment on the hybridization between *Triton palmatus* ♀ and *Salamandra maculosa* ♂ was undertaken by Baltzer (4, 5), who reported that this type of hybrid could not survive longer than the late blastula or the early gastrula stage. A detailed study of the development of this lethal was further carried out by Schönmann (56), according to whom the egg began to show abnormal mitoses at a period of about 23–29 hrs. after fertilization at 18° C. This was named by him as the critical phase of this hybrid. Morphogenetically it corresponds to the late blastula stage.

The lethality of such a combination was later investigated by the method of transplantation. According to Baltzer (5) and Lüthi (49) the implant, which was taken from the animal hemisphere of the lethal hybrid, could develop further, and it took part in the differentiation of various organs and tissues of the host. More recently Baltzer and Schönmann (11) undertook another experiment by carrying out the transplantation at an earlier stage and found that the lethality was autonomous. Attempt has also been made by the two authors to study the nucleic acid contents in the embryonic cells of these lethals. However, owing to the limit of materials, their results in this respect cannot be considered as conclusive and more specimens have been prepared by Baltzer and this writer for further investigation of the nucleic acid problem. As mentioned above, it is of essential importance to know the metabolic activity of this hybrid combination, particularly in its critical phase. Such knowledge will enable us to envisage its developmental anomaly as well as the physiology of lethality as a whole.

A review of the literature on the respiration of the amphibians in the embryonic development has shown that most of the investigators used anurans as the working material (with the exception of Wills (61) on *Triturus torosus* and *Amblystoma maculatum*, and Fischer and Hartwig (29) on *Amblystoma mexicanum*). For the purpose of comparative study, it seems therefore desirable to devote a large part of the investigation on a careful analysis of the controls, i.e. the normal development of *Triton palmatus* and *taeniatus*. This should also contribute to our knowledge concerning the metabolism responsible for the early development of the urodeles.

MATERIAL AND METHODS

As materials for the cross fertilization both *Triton palmatus* and *taeniatus* eggs and *Salamandra atra* sperms were used.[1] The two *Triton* species are very closely related, and there is practically no noticeable difference in using both kinds of eggs. About two days before insemination both females and males were injected with gonadotropic hormone in order to make sure that an ample number of eggs could be found in the uterus and that the sperms were in an active condition. Details of the hybridization technique have been described by Schönmann (56). Right after insemination the eggs were brought to 18° C and the first two cleavages were controlled. Only those eggs which showed normal cell divisions have been used for the measurement of the oxygen consumption.

A capillary respirometer constructed according to the principle of Gerard and Hartline (31) was employed. Since details of handling this apparatus have been given by Chen (22), only those procedures which were modified for the present investigation deserve a full description. All capillaries used had a diameter of about 0.5 mm. The egg, without being taken out of its jelly, was washed several times in sterilized tap-water, carefully inserted into the container, and dried with a small piece of clean filter paper. The oxygen measurements usually lasted one hour although in several cases continuous observation were made even up to four hours. Readings were taken at intervals of 15 minutes, and the experiment was considered to be in order when the shift of the indication drop (20 per cent NaOH) turned out to be regular in the successive intervals. Observations were started only after the temperature of the water bath had come to an equilibrium. This took usually about half an hour. All measurements were carried out at a temperature of $20 \pm 0{,}1°$ C.

Immediately after observation the egg was taken out of the container, transferred into fresh tap water, and kept further at 18° C. For two reasons no fixation was done at the end of observation: 1) the histological picture of these hybrids has been already described in detail by Schönmann and the observation on the living eggs has given us enough assurance of their hybrid nature; and 2) it is rather tedious and difficult to get such hybrids. We have therefore reserved these materials for the further study of the nucleic acids. Nevertheless, in a few cases the eggs were fixed in Zenker's solution soon after measurements, the results of which will be reported in the next section.

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[1] *Salamandra atra* gives birth to fully developed offspring. The development takes place in the uterus of the female, and according to Wunderer (62), it takes about 6–7 weeks until the embryo moves out of the jelly. It is assumed that the complete development lasts about 3 years. This animal has therefore a much slower developmental speed in comparison with *Triton*.

Serving as controls, eggs from the same female but fertilized with sperms of the same species were used. However, owing to the shortage of available eggs in the uterus, eggs laid by the same female in the aquarium before the operation have also been collected for the control experiments. In such cases the first two cleavages were always controlled, and those which showed irregular divisions were immediately rejected. In order to avoid any disturbance due to the experimental condition, measurements of the hybrids and the controls were conducted one after the other.

Since all hybrid and control eggs were measured without taking off the jelly, objections could be raised as to the role played by the jelly itself in the oxygen uptake as well as the possible disturbance caused by the bacteria adhering to its surface. However all the hybridization experiments reported here were carried out under sterilized conditions, and the eggs, as described above, were washed carefully in sterilized tapwater before they were introduced into the apparatus. Furthermore, a control observation was performed by inserting only the jelly into the container. After more than one hour of observation the indication drop in the capillary showed a very slight shift amounting to only 0,008 cu.mm. This is obviously within the experimental error. In this place it should also be pointed out that all oxygen measurements of both the hybrids and the controls were made under exactly the same condition. For the purpose of comparative studies the slight disturbance due to technical difficulties is thus automatically eliminated.

EXPERIMENTAL RESULTS

1. The rate of oxygen consumption in the early development of the controls (*Triton palmatus* and *taeniatus*).

The results of 39 experiments, extending from the 4-cell to the young neurula stages, are summarized in Table I. The developmental conditions of the egg at the beginning and at the end of the measurement are presented in columns 4 and 5 respectively. It can be readily seen that in all these cases development continued within the period of observation. This is especially obvious in the early stages, during which the number of cells can be counted with certainty. Evidently a staying of 2 to 6 hours in the observing container causes no serious interference with the normal development. The rate values of the oxygen consumption can therefore be accepted as giving a real picture of the metabolic activity in the early development of these individuals.

In regard to the rates of oxygen consumption, the values in Table I indicate that a certain individual variation exists. It is a well known fact that eggs from the same female can be of widely different sizes. In the present study the hybrids were derived from 4 individual females. (Four series of cross-fertilization were made: three series with the combination *Triton palmatus* ♀

TABLE I

The rates of oxygen consumption in the early embryonic development of *Triton palmatus* and *taeniatus*.

Exp. series	Egg type	Age in hr. after fertilization (18° C)	Development of egg at beginning of measurement	Development of egg at end of measurement	Oxygen consumption in cu.mm per hr. per egg (20° C)	
1	*Tr. taeniatus*	8	4-cell stage	8–cell stage	0.106	
2	*Tr. palmatus*	8	8-cell stage	16–cell stage	0.110	
3	*Tr. taeniatus*	8	4-cell stage	8–cell stage	0.134	
4	*Tr. palmatus*	9	8-cell stage	16–cell stage	0.135	
5	» »	11	8-cell stage	ø 5–6 cells	0.081	
6	*Tr. taeniatus*	13	16-cell stage	ø 5–6 cells	0.086	Morula
7	*Tr. palmatus*	14	ø 8 cells	ø 13–14 cells	0.089	0.118
8	» »	15	ø 4–5 cells	ø 8–9 cells	0.147	
9	» »	16	ø 10–11 cells	ø 13–14 cells	0.152	
10	» »	18	ø 12–13 cells	ø 22–24 cells	0.142	
11	» »	20	ø 16 cells	ø 18–20 cells	0.153	
12	» »	25	ø 18–20 cells	ø 24 cells	0.158	
13	*Tr. taeniatus*	25	ø 24 cells	ø 26 cells	0.186	
14	*Tr. palmatus*	27	ø 25 cells	ø 27 cells	0.122	
15	*Tr. taeniatus*	27	ø 26 cells	ø 28 cells	0.163	
16	» »	29	Old blastula	Dorsal lip formed	0.111	Blastula 0.150
17	» »	30	ø 30 cells	Old blastula	0.101	
18	» »	33	Old blastula	Dorsal lip formed	0.167	
19	» »	34	Old blastula	Old blastula	0.187	
20	» »	34	Dorsal lip in appearance	Dorsal lip distinct	0.088	
21	*Tr. palmatus*	35	Dorsal lip formed	Dorsal lip distinct	0.179	
22	*Tr. taeniatus*	37	Dorsal lip in appearance	Dorsal lip distinct	0.113	
23	*Tr. palmatus*	40	Dorsal lip formed	Dorsal lip sickle in shape	0.169	
24	*Tr. taeniatus*	41	Dorsal lip formed	Dorsal lip sickle-shaped	0.153	
25	» »	45	Dorsal lip formed	Dorsal lip sickle-shaped	0.163	

(*cont.*)

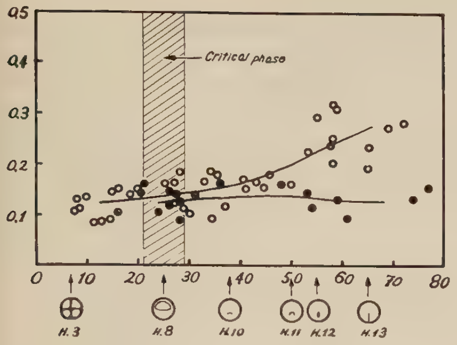
ø refers to the number of cells counted across the diameter of the animal hemisphere.

Experiments 19 and 32 are respiratory measurements conducted on the same egg at the respective time intervals.

Table I (*cont.*)

Exp. series	Egg type	Age in hr. after fertilization (18° C)	Development of egg at beginning of measurement	Development of egg at end of measurement	Oxygen consumption in cu.mm per hr. per egg (20° C)	
26	*Tr. taeniatus*	44	Dorsal lip sickle-shaped	Dorsal lip horse-shoe in shape	0.152	Gastrula 0.174
27	» »	45	Dorsal lip sickle-shaped	Dorsal lip horse-shoe in shape	0.177	
28	» »	50	Dorsal lip horse-shoe in shape	Blastopore slit-like	0.160	
29	» »	53	Small yolk plug	Small yolk plug	0.223	
30	*Tr. palmatus*	57	Old gastrula	Small yolk plug	0.237	
31	» »	58	Old gastrula	Small yolk plug	0.201	
32	*Tr. taeniatus*	58	Old gastrula	Neural groove faint	0.252	
33	» »	55	Blastopore slit-like	Neural groove in appearance	0.299	Neurula 0.273
34	*Tr. palmatus*	59	Old gastrula	Small yolk plug	0.311	
35	*Tr. taeniatus*	59	Blastopore slit in shape	Neural groove faint	0.321	
36	» »	65	Blastopore slit-shaped	Neural groove faint	0.234	
37	» »	65	Blastopore slit-shaped	Neural groove faint	0.190	
38	» »	69	Neural groove faint	Neural plate in appearance	0.274	
39	» »	72	Neural groove distinct	Young neurula	0.284	

× *Salamandra atra* ♂ and one series *Triton taeniatus* ♀ × *Salamandra atra* ♂). One can therefore understand that the results varied to a certain extent. Nevertheless, the trend of the observed data obviously follows an ascending course (Fig. 1). In case the values are grouped according to different developmental stages (see the last column in Table I), the increase of the respiratory rates is even more clear. This point can be further confirmed by the fact that in one case, instead of observing the oxygen uptake on different individ-



Fig, 1.

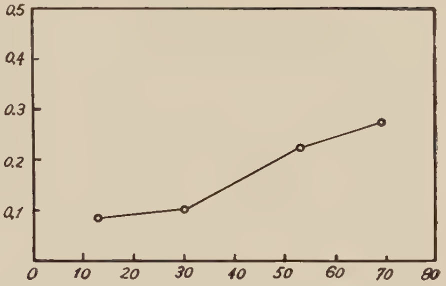


Fig. 2.

Fig. 1. The rate of oxygen consumption during the early embryonic development of the control egg (*Triton palmatus* and *taeniatus*) and the lethal hybrid (*Triton palmatus* or *taeniatus* ♀ × *Salamandra atra* ♂). Ordinate: oxygen uptake in cu.mm per egg per hour. Abscissa: egg age in hours after fertilization at 18° C with indication of developmental stages according to HARRISON's table for *Amblystoma punctatum*. ○ = control egg; ● = hybrid egg.

Fig. 2. Changes in the rate of oxygen consumption at different embryonic intervals of the same egg (*Triton taeniatus*). Ordinate: rate of oxygen uptake in cu.mm per egg per hour. Abscissa: egg age in hours after fertilization at 18° C.

uals, the measurements were taken on the same egg at 4 different intervals. As shown in Fig. 2 its rate values increase steadily. We therefore conclude that there is a regular rise of the rates of oxygen consumption in the early embryonic development of the normals.

2. The rate of oxygen consumption in the development of the hybrids (*Triton palmatus* ♀ × *Salamandra atra* ♂ and *Triton taeniatus* ♀ × *Salamandra atra* ♂).

The respiratory study of the hybrids consisted of 18 experiments on individual eggs ranging from 16 to 77 hrs. after insemination (Table II and Fig. 1). An analysis of the development of these individuals within the period of observation (Table II, columns 4 and 5) shows that in the first 30 hrs. they can still keep pace with the controls of correspondings ages. From 36 hrs. onward they remained in the early gastrula stage and no more development was noted. The observation thus confirmed the early reports of Baltzer (4, 5) and Schönmann (55).

One outstanding feature of the values of oxygen consumption in these hybrids is that they remain constant in both blastula and gastrula stages. (The small difference between the average values presented in the last column of Table II is statistically not significant. p is equal to 0.20.) On the other hand, if the figures of the individuals aged from 16 to 28 hrs. are compared with those of the controls of the same age, the former show distinctly lower values.

TABLE II

The rate of oxygen consumption in the lethal hybrids between *Triton palmatus* ♀ and *Salamandra atra* ♂ (*Triton taeniatus* ♀ and *Salamandra atra* ♂).

Exp. series	Egg type	Age in hr. after fertilization (18° C)	Development of egg at beginning of mesaurement	Development of egg at end of measurement	Oxygen consumption in cu.mm per egg per hr. (20° C)	
1	pat	16	ø 11 cells	ø 14–15 cells	0.104	Blastula 0.125
2	pat	20	ø 18–19 cells	ø 24–26 cells	0.147	
3	tat	21	ø 20–24 cells	ø 28 cells	0.158	
4	pat	24	ø 20 cells	ø 24 cells	0.102	
5	pat	26	ø 18–16 cells	ø 20 cells	0.140	
6	tat	26	Old blastula	Old blastula	0.118	
7	tat	27	» »	» »	0.142	
8	pat	28	» »	» »	0.125	
9	pat	28	» »	» »	0.085	
10	tat	31	» »	Early gastrula	0.140	Gastrula 0.140
11	tat	36	Early gastrula	» »	0.165	
12	tat	48	» »	» »	0.167	
13	pat	53	» »	» »	0.147	
14	pat	54	» »	» »	0.117	
15	tat	59	» »	» »	0.132	
16	tat	61	» »	» »	0.094	
17	tat	74	» »	» »	0.142	
18	tat	77	» »	» »	0.160	

pat indicates hybrids of the combination *Tr. palmatus* ♀ × *Sa. atra* ♂ and "tat" *Tr. taeniatus* ♀ × *Sa. atra* ♂.

The lethal hybrids aged more than 48 hrs. looked pale and usually scattered, degenerating materials squeezed out from egg could be seen within the jelly.

Within this period the hybrids have an average value of 0.126 while the controls are 0.157 cu.mm per egg per hour. (According to t-test p has a value of 0.02.) This corresponds approximately with the critical phase of these lethals which, according to Schönmann (55), occurs at about 23 to 29 hrs. after fertilization at 18° C). The remarkable fact that these lethal hybrids have lower rates of oxygen consumption at the very beginning of their morphological anomaly is especially noteworthy.

DISCUSSION

It is an accepted view that the rates of oxygen consumption rise continuously in the early development of the amphibian eggs.[1] Apparently this is due to the growth of embryonic tissues and the gradual consumption of the less active yolk materials. In the present study the findings based on 39 experiments on the normal individuals of *Triton palmatus* and *taeniatus* are in agreement with this view. However, it seems that the increase of the rate values is more rapid at the beginning of neurulation. As shown in Table I the average value in the neurula stage amounts to about 57 per cent higher than that in the gastrula stage. Most likely at this particular period, there is not only the continuous growth of embryonic tissues but there are also complicated processes such as the induction of the neural plate; and the differentiation of chorda and somites are in progress.

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[1] An entirely different picture in the oxygen consumption during the embryonic development of the sea urchin egg (*Paracentrotus lividus*) has been reported by Lindahl (48). The respiratory

Upon comparing the absolute rates of oxygen consumption with those reported by previous workers on other amphibian species (on *Rana temporaria* (14, 54); on *Bufo vulgaris* and *viridis* (57, 58); on *Triturus torosus*, *Amblystoma maculatum* and *tigrinum*, and *Rana pipiens* (61); on *Rana pipiens* (1); on *Rana pipiens* and *sylvatica* (21); on *Rana fusca* (18)), the values recorded in this study are somewhat lower. In this regard the sizes of the eggs in these two urodeles should be taken into consideration. According to the determination of 12 eggs of different developmental ages the moist weight on the average amounts to 2.00 ± 0.17 mg. The dry weight, based on 6 determinations, has an average value of 0.61 ± 0.02 mg. If we adopt the weight as a basis of comparison, the rate values observed here turn out to be relatively high. Since no extensive data on the amount of inert yolk in the egg of each species are available, and the developmental condition of the egg varies from individual to individual, an exact comparison between the respiratory rates of even closely related organisms is extremely difficult.

Experiments performed on the hybrids are not as extensive as that on the controls. However, the results of 18 observations have provided us with a general picture of the metabolism of these lethals from shortly before the first appearance of cytological abnormality until the failure of further development, and finally the degeneration of a large part of the embryonic cells.

According to the report of Schönmann's investigation ((56) p. 364, Table 7), the lethal effect begins with abnormal nuclei and mitotic figures in the old blastula (about Harrison's stage 8^{+}). A careful examination of the present results shows that within this period, i.e. 23–29 hrs. after fertilization, the respiratory rates of the lethal hybrids are on the average 18 per cent lower than those of the normals. We therefore conclude that in the critical phase, according to Schönmann's nomenclature, the hybrids show not only cytological abnormality, but also metabolic weakness. Since no respiratory measurements have been made in earlier stages, we have no means to ascertain whether the tendency leading to a lowering of the metabolism is underway even before the critical phase.

Because of the many complications and unknown facts in embryonic metabolism, it is at the present moment not easy to ascertain the immediate cause which leads to the reduction of the respiration. We have even no idea about the causal relationship between the two phenomena which characterize the critical phase of this hybrid, i.e. nuclear abnormality and metabolic weakness. Presumably the nucleus, with half of

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curve is first S-shaped, then takes an abrupt ascent, and finally changes into a nearly horizontal line corresponding to a steady but very small increase in the last stage. As has been discussed by him, the metabolic process and the morphogenetic development are different at these three periods.

its material coming from a widely different form, disturbs in a certain way the normal function of the enzyme system. Brachet (20) has pointed out that in the lethal hybrid between anurans the development is usually arrested at the late blastula or at the early gastrula stage, during which important morphological and biochemical changes in the nuclei take place. He also suggested the possibility of a block in the synthesis of ribonucleic acid and proteins in such lethals. Furthermore, Barth and Jaeger (13) have shown that lethal hybrids of the combination *Rana pipiens* ♀ × *Rana sylvatica* ♂ seem to be unable to maintain energy-transferring substances such as ATP (and possibly creatine phosphate) in a phosphorylated condition. Other important physiological processes like the production of lactic acid (12) and the utilization of carbohydrate (33) have also been reported to be lower in the hybrids than in the normals. The results of these studies furnish us valuable information concerning the anomaly involved in such developmental interruption.

The fate of the present lethal eggs, according to the description of Schönmann, goes on as follows: both abnormal and pyknotic nuclei increase steadily in percentage; the dead cells are squeezed into the blastocoel; and finally only the materials in the neighbourhood of the dorsal lip are saved from the degeneration process and can remain as a mass of tissue without further differentiation for a period of as long as 121 hours. Concerning the failure of further gastrulation Schönmann suggested 3 possible causes: 1) mechanical hindrance due to dead cells in blastocoel; 2) loss of too much material; and 3) the damage of invaginating cells due to poisonous substances derived from the degenerating tissue. The present study indicates clearly that the rates of oxygen uptake of the lethals remain about the same in the later development, while those of the normals keep on rising. Similar phenomena have been observed by Barth (12) in the lethal hybrid between *Rana pipiens* ♀ and *Rana sylvatica* ♂. According to his results the rate of oxygen consumption is the same in both normals and hybrids up to the early gastrula stage. Later on the value increases in the normals but it remains the same in the lethals. It could be possible that the metabolism of the hybrids is affected to such an extent that it is no more capable to meet the demand for further development. In case this is true, we find in this place another explanation for the developmental standstill. However, this view should be accepted cautiously, because, as pointed out previously, such a metabolic weakness can be only a secondary phenomenon. Further experiment is needed for the clarification of this point.

The fact that the embryonic material in the lethals keeps on deteriorating while the respiratory rate maintains the same value in individuals aged even more than 70 hrs. requires an explanation of its own. As Schönmann pointed out, in all these hybrids it is always the dorsal lip region which is least damaged by the lethal effect. In the present study the sections of two such lethal hybrids fixed more than 70 hrs. after fertilization also showed that most of the cells in the neighbourhood of the dorsal lip were practically normal. In another experiment (Baltzer and Chen, unpublished) it has been observed that materials from the dorsal lip region, after being inserted into the blastocoel of a normal egg, were still capable to induce a secondary embryo. The transplantation experiment of Lüthi (48) indicated clearly that implants taken from the animal half of such aged hybrids developed further and took a part in the differentiation of many organs of the host. The results of all these observations have therefore revealed the same fact that a certain part of this lethal hybrid, mainly the dorsal lip region, was relatively healthy and able to assume further development if an adequate environment was given. The work of Brachet and Shapiro (21) on *Rana sylvatica* demonstrated that the rate of oxygen consumption in the dorsal lip hemisphere was 45 per cent higher than that in the vegetative hemisphere. A similar conclusion was reached by the study on *Amblystoma mexicanum* by Fischer and Hartwig (29). The two authors reported that the respiration in the animal hemisphere amounted to 3.1 times higher than that in the vegetative hemisphere, and the rate of oxygen uptake was 20 per cent higher in the dorsal lip than in the ventral ectoderm. We see immediately that it is that particular part in the lethal hybrid which shows the highest metabolic activity saved from the lethal effect. An abrupt decrease of the rate of oxygen uptake is therefore not expected. This could be an indirect confirmation of the findings reported by the authors mentioned above.[1]

Finally a comparative analysis should be given to the respiratory aspects observed in the present hybrid and those recorded in the lethal mutants of *Drosophila melanogaster* (22). Such an analysis might bring us a step closer towards the solution of the problem of lethality. As pointed out at the beginning of this report, the immediate causes of these two types of lethals are entirely different: the former is due to the unfitness of the heterogeneous nuclear material, while the latter is due to a genic change in the chromosome of one species. However, the results of studies on the oxygen consumption in both groups have shown that they are similar in the following aspects:

Firstly, in both groups there are certain important developmental stages which are especially sensitive to the lethal effect. For the hybrids these are in general the first cleavage processes, the gastrulation, and the formation of essential organs, cf. Hertwig (46). For the lethal mutants, at least in *Drosophila*, they are the early embryonic development, embryonic hatching, puparium-formation and imaginal hatching, cf. Hadorn (41). The explanation is that in all these periods important developmental changes occur and special energy is required. In accordance with this view the hybrid in the present case fails to develop at the beginning of gastrulation although according to Schönmann its critical phase already starts in the late blastula stage. In any case the present study showed that throughout this period the hybrids never reached the limit of oxygen uptake found in the controls of corresponding ages. For the three lethal mutants in *Drosophila*, further development is retarded at the third larval instar (*lme*) or at the time of switching over from larval to pupal life (*lgl* and *ltr*). Again the respiratory rates of these lethals suffer a distinct drop at these periods. Therefore, in both lethal groups we find exactly the same fact: the impairment of the metabolic activity results in the shortage of energy which is required for overcoming important developmental processes in a critical phase.

Secondly, in later stages of both lethal types the rates oxygen consumption in all individuals remain low, and their morphogenetic development is at the same time restricted. Thus the development of the hybrid stops in the early gastrula stage, and the lethal mutants continue their life as aged larvae or pupae. Even in the case of the partially metamorphosed *ltr*/*ltr* pupae, their morphogenetic differentiation never reaches the extent of a normal individual. The drop of metabolic rate does not mean the delaying, but the ceasing of normal development. It seems that in order to attain the normal morphological differentiation a minimal limit of metabolism is necessary. In other words, there is a close relationship between respiration and development. This principle holds true in both types of lethals.

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[1] By the method of vital staining results in favour of the dorsal lip region have been obtained by Fischer and Hartwig (29) and Piepho (55). However Boell and Needham found no significant difference in the oxygen consumption between the dorsal lip and the ventral ectoderm (see literature in Needham (53)). It is highly doubtful if the isolated explant behaves normally as in an intact egg. This problem cannot be considered as settled (see also discussion in Lehmann (47) p. 341).

Thirdly, workers dealing with lethals used to set the end of life in such individuals at the moment when their apparent development comes to a standstill. The observation of the metabolic rate in both experiments shows that such a criterion should be accepted carefully. The lethals can possibly survive a much longer period than their apparent growth shows. The lethal effect does not bring about a sudden death to such an individual in a dramatic way. There are always tissues and organs which are able to withstand the lethality and remain alive even after the death of the other parts.

SUMMARY

1. The results of 39 experiments performed on *Triton palmatus* and *taeniatus* indicate that there is a continuous rise of respiration in the early embryonic development. Such an incrase is slower until gastrulation, and more rapid at the end of gastrulation and during neurulation.

2. The lethal hybrids of the combination *Triton palmatus* ♀ × *Salamandra atra* ♂ or *Triton taeniatus* ♀ × *Salamandra atra* ♂ show a lower rate of oxygen consumption than the controls in the critical phase, i.e. in the advanced blastula stage. In later stages the respiratory rate remains low and the development does not exceed the early gastrula stage.

3. Based on the results of the present study and those observed on the three lethal mutants in *Drosophila melanogaster*, i.e. *ltr*, *lgl*, and *lme*, the general aspects in the metabolism of lethality are discussed.

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DESOXYRIBONUCLEIC ACID IN *ARBACIA* EGGS[1]

A. MARSHAK and CELIA MARSHAK

Marine Biological Laboratory, Woods Hole, Mass., U.S.A.

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In recent years there have appeared a number of reports giving the amount of desoxyribonucleic acid (DNA) in eggs and sperm of sea urchins and other forms. Altho various methods have been used they have all been inadequate for the material to such an extent that even the order of magnitude of the amount of DNA per egg remains uncertain. Of particular importance is the problem of how much, if any, of the DNA found in these analyses is contained in the nucleus and how much in the cytoplasm. In striking contrast to the large amounts of DNA reported to be in the egg (3, 6, 13) is the observation briefly noted in a previous publication that the nucleus of the mature egg fails to give a positive Feulgen reaction (9). The present paper gives DNA values determined by the method of isotope dilution (12) which is considered to be free of the errors associated with procedures previously used and reports additional observations which may account for the high DNA values obtained in the past.

METHODS

C^{14}-thymine. Thiobacillus thioparus isolated from marine mud was cultured in a liquid mineral medium of the following composition to which CO_2 was added as the sole carbon source.

10 g $Na_2S_2O_3 \cdot 5\ H_2O$, 4 g KH_2PO_4, 0.8 g $MgSO_4 \cdot 7\ H_2O$, 0.2 g $(NH_4)_2SO_4$, 10 ml metal solution (see below) and distilled water to 1 liter.

Metal solution: 5.0 g ethylenediamine tetraacetic acid ("Versene" free acid), 0.554 g $CaCl_2$, 2.20 g $ZnSO_4 \cdot 7\ H_2O$, 1.144 g H_3BO_3, 0.506 g $MnCl_2 \cdot 4\ H_2O$, 0.499 g $FeSO_4 \cdot 7\ H_2O$, 0.110 g $(NH_4)_6\ Mo_7O_{24} \cdot 4\ H_2O$, 0.157 g $CuSO_4 \cdot 5\ H_2O$ and 0.161 g $CoCl_2 \cdot 6\ H_2O$.

Added 50 ml H_2O (distilled) and heated to boiling. With KOH adjusted to pH 6.5–6.8 and volume made to 100 ml.

One liter of the medium in a sterile 3 liter heavy wall pyrex erlenmeyer flask was inoculated with bacteria in the log phase of growth. The air in the flask was then evacuated through a manifold and the $C^{14}O_2$ generated by reacting $BaC^{14}O_3$ with HCl introduced. Oxygen was then allowed to flow into the vessel until the pressure equalled atmospheric pressure. The sealed vessel was then incubated with gentle shaking at room temperature (25–28° C) for 3 days. The bacteria were killed by heating the culture to 70–80° C and the gas of the culture flask evacuated thru $Ba(OH)_2$ traps. The bacteria were collected by centrifugation and extracted twice with cold 5 per cent trichloracetic acid after which lipids were removed by extraction with cold 70 per cent ethanol, cold 95 per cent ethanol, hot ethanolether (3 : 1) and hot petroleum ether, the latter two extractions being carried out under reflux. The weight of the residue after drying in a vacuum desiccator was approximately 300 mg. The residue was then subjected to repeated freezing and thawing to obtain some degree of homogenization. It was then extracted with 5 per cent trichloracetic acid at 90° C three times to remove nucleic acids (15). The extract was made 1 N with HCl and heated at 100° C for 1 hour to liberate the purines after which the solution was neutralized and subjected to the countercurrent separation procedure of Tinker and Brown using butanol and water (18). The adenine and guanine were contained in plates 0–11 and the pyrimidine nucleotides in plate 12. After being taken to dryness, the pyrimidine fraction was digested with 70 per cent perchloric acid (10). After precipitation of the perchloric acid as the potassium salt, the hydrolysate together with the butanol eluate of the salt was subjected to anion exchange chromatography using Dowex 1 as described by Cohn (2). Before being applied to the column the solution was taken to dryness, extracted with butanol after which the butanol extract was dried under vacuum and the residue taken up in a small amount of dilute NaOH. This procedure was found necessary in order to eliminate salts, probably perchlorates, which interfered with the retention of the pyrimidines on the column. The amount of thymine isolated was approximately 2.5 μmols. The thymine so obtained was found to be 95 per cent pure by chromatography on paper, i.e. only 5 per cent of the radioactivity was outside the thymine spot on the paper chromatogram. For use in experiments with the *Arbacia* eggs this product was then subjected to paper chromatography and the thymine collected by elution with butanol. This thymine was found to be pure spectrophotometrically and to be at least 98 per cent pure with respect to C^{14}.

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[1] Supported by a grant from the Division of Biology and Medicine of the Atomic Energy Commission.

To a solution containing 1.65 μg of the C^{14}-thymine recovered from the bacteria, 350.5 μg of inactive thymine was added and two aliquots of the mixture chromatographed on paper. Spectrophotometric analyses of the eluates gave values of 49.6 and 50.1 μg indicating an accuracy of 1 per cent for the method. Aliquots of the solution were also deposited with a Gilmont ultramicroburette[1] on metal planchets previously coated with isobutylmethacrylate polymer and a thin film of silicone stopcock grease. The area in the center of the planchet received 0.04 ml of a 1/200 aqueous solution of polyoxyethylene sorbitan monooleate which was evaporated to dryness before the radioactive solution was deposited. This procedure defined the area over which the radioactive solution spread when dried under an infra-red lamp. The accuracy of the procedure for depositing the aliquots and for measuring the amount of C^{14} present was determined with a sample of C^{14}-glycine containing 1 mg glycine per ml. Two planchets each received 0.32 ml of this solution and gave counts agreeing within less than 0.5 per cent. Measurements were made with a Q-gas counter.[2]

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[1] Obtained from Atomlab. Co., N.Y., N.Y.

[2] Made by Emil Greiner and Co., N.Y., N.Y.

A total of 10 000 counts were taken with each sample and counts were greater than 100 times the background.

Eggs. For "DNA" analysis eggs were obtained from 25 females. Shedding of eggs was stimulated by addition of 3–4 drops of 0.5 M KCl to the coelomic cavity. The shed eggs were suspended in filtered sea water and then filtered through bolting cloth (160 treads to the inch). They were then washed 5 times in filtered sea water in six large crystallizing dishes by allowing the eggs to settle by gravity and siphoning off the supernatant fluid. Only $^3/_4$ of the suspension obtained was used in these experiments. They were extracted with 450 ml acetone-ethanol (2 : 1) for 1 hour and then 3 times in boiling acetone-ethanol under reflux for $^1/_2$ hour each time after which they were extracted in chloroform-methanol (1 : 1) overnight at room temperature and stored in acetone-ethanol.

Before proceeding with further extraction, counts of the number of eggs in the suspension were made by taking up appropriate dilutions into wide bore 1 ml pipettes graduated in 0.1 ml which were immediately placed in a horizontal position and stoppered at each end. Eleven such aliquots were counted, and the number of eggs in the suspension found to be $10.5 \times 10^7 \pm 0.37 \times 10^7$, the latter number being the standard deviation.

After removal of the acetone-ethanol, the eggs were treated by the method of Schmidt and Thannhauser (14) to obtain the DNA in an insoluble residue and the ribonucleotides in a soluble fraction. In applying this procedure, digestion in N KOH was at 30° C and not at 37° C as described in the original method. Another modification was a preliminary extraction with petroleum ether under reflux to remove substances found previously to interfere with perchloric acid digestion (10). The DNA-containing residue was washed 3 times in cold 0.125 N trichloracetic acid and dried to constant weight in an Abderhalden apparatus at 100° C.

Sperm. Sperm were collected and washed as previously described (10). The sperm suspension was placed in tared centrifuge tubes and the supernatant sea water removed after centrifugation for 30 minutes at 1 500 g. The sediment was then extracted as previously described to remove lipids and acid soluble materials and dried to constant weight (10). Appropriate dilutions of aliquots of the sperm suspension were counted in a Levy-Neubauer haemocytometer. Approximately 100 000 cells were counted and the standard deviation was 0.5 per cent.

RESULTS

RNA. The fraction containing the RNA nucleotides was neutralized with cold $HClO_4$ and the precipitate removed by centrifugation. The supernatant fluid together with the precipitate washings was taken to dryness in a stream of dry air at 70° C. It was then taken to constant weight in a vacuum desiccator over P_2O_5. An aliquot (115 mg) was digested with perchloric acid, and chromatography carried out on paper as previously described (10). After precipitation of the perchloric acid as the potassium salt and concentration by evaporation, aliquots of 62.4 μl were deposited as single spots on paper strips which were then developed with the butanol-HCl system. Several such spots after development showed no thymine detectible on the paper with the ultraviolet lamp and elution of appropriate areas as indicated by control thymine spots on the same chromatogram showed no significant absorption in the Beckman spectrophotometer. Appreciable amounts of uracil were obtained, i.e. an average of 8.5 μg per spot, from which it was calculated that a single egg contained 9.25×10^{-4} μg of uracil. From data previously presented (10) it was calculated that there were 8.45 mols of nitrogenous base per mol of uracil in *Arbacia* egg RNA. Using this factor and a mean nucleotide weight of 334 g/mol (obtained as a weighted mean) it was calculated that there were 24.4×10^{-4} μg RNA per egg or 21.6×10^{-5} μg RNA-phosphorus per egg. This compares well with the value 20×10^{-5} μg RNA-phosphorus per egg given by Schmidt *et al.* (13).

DNA. The insoluble fraction containing the DNA was taken to constant weight (2.000 gms) in an Abderhalden apparatus. An amount weighing 1.2678 gms was digested with perchloric acid and the C^{14}-thymine solution added immediately afterwards. The mixture was stirred for 30 minutes at room temperature. The undissolved residue was removed by centrifugation and the chilled supernatant neutralized (pH 6.9) with cold KOH and stirred in the cold for 30 minutes after which the precipitate was removed by centrifugation. To the combined supernatant and washings from the precipitate approximately 500 mg of Norit A was added and the mixture stirred at room temperature for an hour. The Norit was then extracted with 1 ml of redistilled butanol for 30 minutes and the volume of butanol reduced in a stream of dry air to 0.3 ml. Two aliquots of 106.12 μl were chromatographed on paper with the butanol-HCl system, each chromatogram containing a standard thymine spot in one lane as an index of the expected position of thymine. Each of these chromatograms on examination with the ultraviolet light showed a very obvious spot in the lane containing the digest corresponding in position to the "standard thymine" spot. The former were then eluted with 0.1 N HCl, the eluates combined, the HCl removed by vacuum distillation at 50–60° C, the residue then being taken up in butanol and rechromatographed in the butanol-ammonia system. The developed chromatogram showed strong ultraviolet light absorption at the level for thymine. In this chromatogram the blue fluorescence observed in the previous ones between the starting line and the thymine in the digest lane was now absent. The thymine in the spot was eluted with 0.1 N HCl and the absorption curve of the eluate determined in the spectrophotometer. The absorption curve was in good agreement with that for pure thymine only in the region

280–260 mμ. This solution was again subjected to chromatography in butanol-HCl. The eluate obtained from this chromatogram was in good agreement (within 1 per cent) with the standard curve in the region 280–245 mμ and the thymine was therefore considered to be sufficiently purified for quantitative estimation from the spectrophotometric data. Calculation showed that the eluate contained 7.20 μg thymine which could be determined with an accuracy of 2 per cent. The radioactivity in this solution was then determined and 2 aliquots of the C^{14}-thymine solution originally added to the digest counted at the same time. A total of 20 000 counts was taken with each sample. The counts for the thymine recovered from the digest were more than 10 × background. Thymine present in the digest was calculated as follows:

$x =$ μg thymine added to digest $= 252.45$ μg, $y =$ μg thymine already present in the digest, $S_i =$ initial specific activity $= 234.5$ cpm/μg and $S_f =$ final specific activity $= 192.4$ cpm/μg

$$S_i/S_f = x + y/x$$
$$y = 56.1 \ \mu g.$$

Since the dry material digested was derived from 67×10^6 eggs, this value represented 8.4×10^{-7} μg thymine per egg. From the ratio of the mols of thymine to mols of desoxyribonucleotide obtained from data previously published (10) for *Arbacia* sperm DNA (0.103), it was computed that there was 8.1×10^{-6} μg DNA per egg.

DNA in sperm. From the dry weight of the lipid-free, acid-extracted residue and the sperm counts, it was calculated that there was 3.07×10^{-6} μg of this material per sperm. Analyses previously reported (10) showed that thymine content of this material was 2.68 per cent. From these values it was found that there was 8.2×10^{-8} μg thymine or 7.9×10^{-7} μg DNA per sperm.

CYTOLOGICAL OBSERVATIONS

Suspensions of extruded eggs were filtered and washed as indicated in the section on methods. After extraction of the pigment and of the lipids, they were stained by the Feulgen methods as previously described (9). In spite of the precautions taken all of the suspensions examined were found to be contaminated with material that contained an abundance of Feulgen positive material. Immature ova were found enmeshed in masses of small cells with small but strongly positive nuclei (Figs. 1, 3). More or less spherical masses of such cells in sizes varying from 1/5 to 2 times the size of the ovum were found, the smaller of which contained no oocytes or oogonia but were made up entirely of masses of these small cells which have been described previously as syncitial cells, nurse cells or Wanderzellen (7, 8, 11, 17). Occasionally a small portion of the ovary was found in which the ova could be seen embedded in the mass of syncitial cells (Fig. 2).

Suspensions of eggs were made by placing the ovaries in sea water and filtering thru 3 layers of cheese cloth. Observations made with the phase contrast microscope on such suspensions showed how the spherical masses mentioned above might be formed. From the ruptured ends of ovarian lobes found in the suspension, elongated ova were extruded into the sea water, many of them going into the polar body divisions as they rounded up. Enveloping some of the extruded eggs, particularly those in the germinal vesicle stage was an envelope of syncitial cells. The latter were embedded in a viscid hyaline mass which in many places rounded up into spheres with the syncitial cells around the surface of the sphere (Fig. 5, 6). In the Feulgen stained material, oocytes whose diameters were 20–23 μ, the chromatin appeared as bright red paired filaments distributed about the periphery of the nucleus. In the later stages with nuclear diameters of approximately 26 μ, the chromatin appeared as short red strands grouped about the nucleolus. Where the nuclear diameter was 27–28 μ, color was detectible only as minute bright red granules distributed about the periphery of the nucleolus. This observation is very similar to that described by Gothié and Tsatsaris (4) for the mouse oocyte. In nuclei larger than those mentioned above no color, localized or diffuse, could be detected. Measurements of pronuclei of mature eggs gave an average diameter of 9.7 μ. Thus oocyte nuclei whose diameters were more than twice that of the pronuclei and whose surface areas were therefore 4 times greater exhibited a pronounced Feulgen stain. However, no color could be seen in the resting pronucleus. The absence of color was particularly striking since the chromatin strands of the pronuclei appeared coarser than those of the meiotic prophases which did stain (Fig. 4). The chromosomes of the meiotic metaphase and anaphase were bright red as was the chromatin of the polar body nucleus. Altho the polar body nuclei were bright red, the nuclei of the ova to which they were attached were completely colorless.

The following experiments were carried out as controls on the staining procedures:

a. The lipid extracted eggs were suspended in 10 ml of a solution containing 0.1 M phosphate buffer at pH 7.0, 0.003 M $MgCl_2$ and 1 mg crystalline DNAse and incubated at 30° C for 90 minutes. Eggs suspended in a similar solution containing no DNAse were incubated at the same time. Both were then stained by the Feulgen procedure. In the latter group, the nuclei of the syncitial cells were deeply stained and nuclei of oocytes in the early meiotic prophases were also stained but not as deeply. None of the cells in the enzyme treated preparations showed any color.

b. Another aliquot of this egg suspension was hydrolyzed with N HCl for 12 minutes at 59–60° C but not stained. On examination they were found to contain no red granules and none of the cell structures were colored with the exception of some coarse yellow granules found associated with some masses of syncitial cells.

c. An aliquot of this suspension unhydrolyzed and unstained appeared as in *b*.

d. An aliquot was treated with the Schiff reagent without hydrolysis. Eggs were pink with nuclei having no more intense color than the cytoplasm. Some of the ovarian fragments with syncitial cells contained coarse yellow granules, others were clear and neither had any red pigmentation.

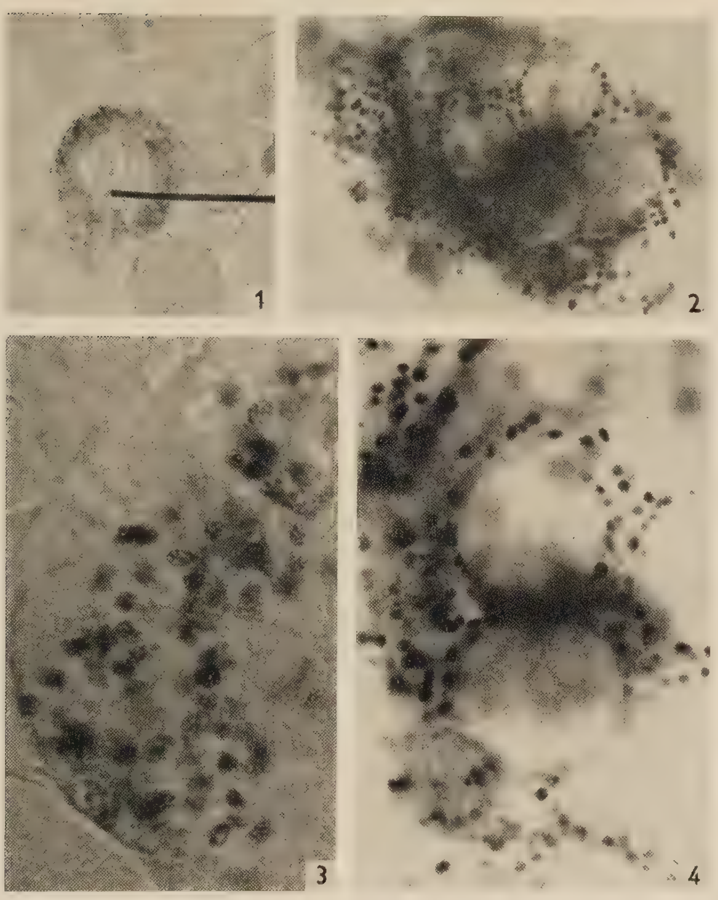
It may be concluded from these experiments that the red color observed in the nuclei of the syncitial cells and those of the young oocytes after Feulgen treatment was due to the DNA contained within them.

Mature unfertilized eggs were suspended and stained together with eggs in the early cleavage stages. Chromosomes in the metaphase and anaphase of the first and subsequent cleavage divisions were bright red. The interkinetic nuclei of the 2 cell stage were a faint but definite red and a progressive increase in intensity of the stains was observed in the nuclei of the successive cleavage divisions. No color could be found in the nucleus of the ripe unfertilized egg. In all cases the cytoplasm was colorless. The diameter of the nucleus of the mature unfertilized egg was not significantly different from that of nuclei of the embryo thru the 16 cell stage (5).

DISCUSSION

Since the extractions which preceded the perchloric acid digestion of the eggs removed all possible low molecular weight thymine compounds, it may be assumed that the thymine found was derived from nucleic acid.

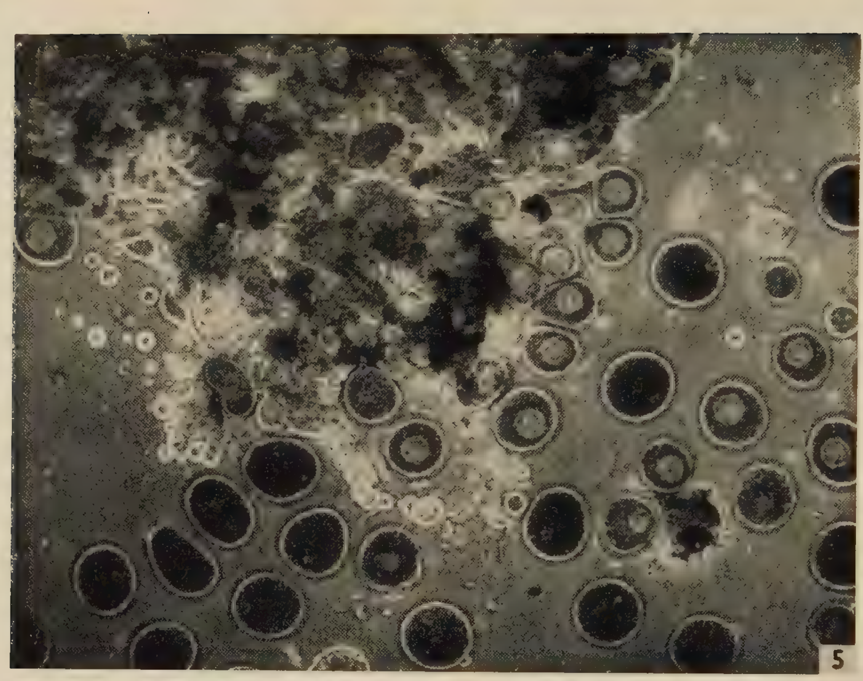
A much greater amount of DNA per egg than the amount per sperm has been reported by several investigators (13, 3, 6). In the first instance, DNA was estimated from phosphorus determinations on the insoluble residue obtained after KOH digestion, an estimate which may be in serious error because of the presence of large amounts of non-DNA materials which may contain phosphorus, as for example phosphoproteins. Their value for DNA per *Arbacia* egg is 5 times that obtained in the present experiments. The bioassay technique used by Elson and Chargaff (3) was recognized as



The material for figures 1–4 was obtained from suspensions of extruded eggs fixed in 5 per cent citric acid and stained by the Feulgen procedure. Figs. 5 and 6 were obtained from living material examined with the phase contrast microscope.
Fig. 1. An immature egg enclosed in an approximately spherical mass containing many Feulgen positive bodies.

Fig. 2. Several immature eggs surrounded by Feulgen positive bodies.
Fig. 3. The Feulgen positive bodies under high magnification showing their nuclear structure.
Fig. 4. Immature ova under high magnification showing the Feulgen positive chromatin of the egg nucleus. Notice the large size of the nucleus relative to the size of the ovum.

being unreliable by these authors particularly at the low concentrations found in the *Paracentrotus* egg suspensions. They report a DNA content per egg which is approximately 2–3 times the amount observed in *Arbacia.* Hoff-Jørgensen and Zeuthen using a microbiological procedure specific for desoxyribose linkages have found a huge amount of this material in the



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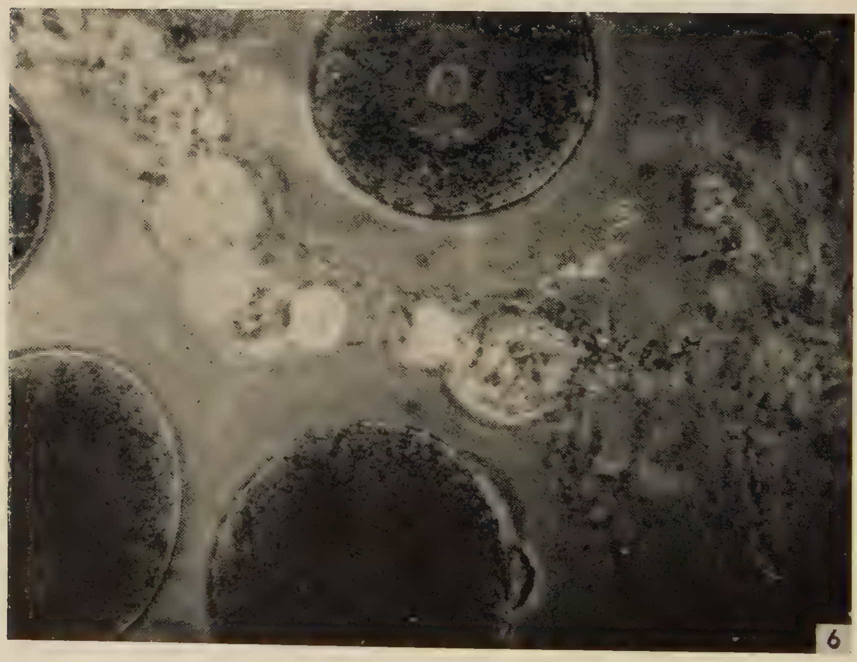

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frog's egg which if converted to the equivalent amount of DNA could not possibly be contained in the egg nucleus. They also made separate determinations on nuclei isolated from these eggs and it seems particularly significant that the DNA content of these nuclei was below the limit of sensitivity of their method. Thus altho large amounts of DNA have been found in chemical analyses of egg suspensions there is no chemical evidence of DNA in the nuclei of these eggs.

The cytological observations here reported indicate that no significance can be assigned to the amount of DNA per egg determined chemically or by bioassay unless the amount that must be attributed to cells other than the egg cells, eg. syncitial cells, follicular cells, polar bodies can be estimated.

If a single fixed amount of DNA per haploid complement of chromosomes (X) is assumed, the amount expected in an egg suspension due to the egg and polar body nuclei is 4 times that amount. Altho the nucleus of the ripe egg is Feulgen negative it may be assumed that the DNA of the chromosomes which formed it is not absent but has been released to the cytoplasm. Similarly the DNA seen in the early meiotic prophases of the egg must also have been given up to the cytoplasm, thus contributing an amount equal to 2 X, making the total 6 X. The amount of DNA found in these experiments (10 X) suggests either a contribution of DNA to the egg cytoplasm from sources outside the egg such as syncitial cells or contamination of the suspension with cells other than egg cells. Counts made of the frequency of the DNA-containing particles of ovarian tissue in the egg suspensions indicated that they were about 1 per cent of the number of eggs. However each such particle contained on the average 100–200 syncitial cells i.e., 1–2 syncitial cells per egg. If each syncitial cell may be assumed to be diploid and have the 2 X amount of DNA, an additional amount of DNA is expected in the suspension, attributable to this source which will amount to 2 X–4 X. Thus the total amount of DNA per ovum expected in the suspension was 8 X–10 X the amount per sperm which approximates the amount found.

The results with the Feulgen stain show that there is a loss of DNA by

Fig. 5. A ruptured portion of an ovarian lobe showing extruded ova some of which have formed polar bodies[1] and the hyaline spheres of viscid material. The irregular dark masses in the ovarian tissue are aggregates of bright red pigment. Notice that some of the viscid material with its embedded granules adheres to the immature ova (lower right).

Fig. 6. Higher magnification of the hyaline spheres showing embedded dark granules. The latter are the Feulgen positive nuclei of the syncitial cells.

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[1] The polar bodies are formed within a few minutes after the ova are extruded into sea water.

the egg nuclei during the meiotic process. There is similar evidence in the early literature from the staining reaction to acid and basic dyes. Jenkinson reported that in *Paracentrotus* the young oocyte nucleus was basophile becoming acidophile in the course of oocyte development and in the subsequent stages of the ripening of the egg the chromatin of the nucleus remained acidophile (7). The absence of stain in the nucleus in the late germinal vesicle stage might be explained by dispersion of the DNA over a relatively huge area or volume. But this explanation cannot be advanced for the absence of stain in the nucleus of the ripe egg. The latter has a diameter less than $^1/_2$ that of the oocytes whose chromatin was obviously stained, or a surface area which is less than $^1/_4$. Thus if it is assumed that the amount of Feulgen-stainable chromatin is a fixed multiple of the chromosome complement, stained chromatin should be visible in the nucleus of the mature ovum if it could be detected in the oocytes mentioned, even if the latter were assumed to contain 4 times the amount of chromatin present in the former. However, this was clearly not the case. It should be pointed out that argument on the basis of the area of possible dispersion is an oversimplification which is not justifiable in this instance. The chromatin is not uniformly distributed over the nuclear surface but exists as definite strands, not only in fixed material but in living cells examined in ultraviolet light. Criteria as to detectability should then be more reliably based on the diameter of the chromatin strands. Since the chromatin of the mature ovum is distinctly coarser than that of the oocytes mentioned, they should have been bright red if they contained DNA. It is clear that on either basis ie., the diameter of the strands or of the nucleus, the Feulgen stain gives no evidence of the presence of DNA in the nucleus of the mature ovum.

Both the analytical data and the cytological observations lead to the same conclusion. The data on frog ova presented by Hoff-Jørgensen and Zeuthen (6) suggest that this situation may not be limited to the *Arbacia* egg. The cytological observations of Serra (16) on eggs of snails and frogs suggest that this condition may be a rather general one altho the possibility of dispersion beyond the level of microscopic detectability has not been eliminated in these species. Alfert (1) has reported that the pronucleus of the mouse egg contains DNA in amounts equivalent to $^1/_4$ to $^1/_2$ that found in the primary oocytes. However, in this species, the female pronucleus is not formed until after fertilization so that a well defined resting stage of the pronucleus comparable to that in *Arbacia* may not exist. In the pronuclei described by Alfert (1) as containing measurable amounts of DNA, the synthesis of new DNA may already have been initiated.

The observations given above lead us to the conclusion that there is no detectable DNA in the pronucleus of the *Arbacia* egg even tho it should have been clearly discernible by the methods used if it were present. The apparent loss of DNA from the chromosomes of the *Arbacia* follows a pattern analogous to that of chromosomes containing heterochromatin ie., stainability is lost first in those portions distal to the regions associated with the nucleolus, but eventually all stainability is lost. At a later time in ontogeny, DNA appears in association with chromosomes or regions thereof which in the unfertilized egg lacked DNA and in the case of *Arbacia* such new DNA cannot be presumed to have been transported to the new sites from other chromosomal regions where it was synthesized from preexisting DNA. That new DNA need not arise from preexisting DNA has been shown in turnover experiments with cells in active mitosis where it was demonstrated that P^{32} appeared in the DNA only after it had first been incorporated into the nuclear RNA.

These observations together with the fact that *Arbacia* eggs may be induced to develop parthenogenetically lead us to the conclusion that DNA must be a derived product and that it cannot be assumed as it has in the past, that DNA is a prime originator of the reactions occurring at specific loci that are assumed to be essential gene functions.

SUMMARY

1. The amount of thymine in a suspension of *Arbacia* eggs was determined by isotope dilution using C^{14}-labelled thymine. The eggs were found to contain 8.4×10^{-7} μg thymine or 8.1×10^{-6} μg DNA per egg.

2. From determinations of uracil it was calculated that there was 2.4×10^{-3} μg RNA per egg.

3. From the thymine content it was determined that there was 7.9×10^{-7} μg DNA per sperm.

4. A portion of the DNA found in the egg suspension could be attributed to aggregates of syncitial cells which were not removed by careful filtration or washing. The amount of DNA from this source was estimated as 2 X–4 X where X is the haploid amount found in the sperm. It was possible to account for the remaining 6 X in the egg suspension as polar body DNA (3 X) and cytoplasmic DNA derived from the egg (3 X).

5. The nucleus of the mature ovum contains no detectible Feulgen-positive material. This deficiency cannot be ascribed to dispersion of DNA over a large volume or area of the nucleus since the nucleus is smaller than that of the immature ovum which has distinctly Feulgen-positive chromatin.

6. Because of the absence of DNA in the nucleus of the mature ovum which is capable of embryological development following parthenogenetic stimulation it is concluded that DNA must be a derived nuclear product and not a prime initiator of gene reactions.

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A DOUBLE-SLIT INTERFEROMETER FOR THE DETERMINATION OF THE THICKNESS OF THE REFERENCE SYSTEM USED IN X-RAY MICRORADIOGRAPHY

E. DJURLE and O. HALLÉN

The Optics Laboratory, Royal Institute of Technology, Stockholm, and Department of Histology, Medical School, Göteborg, Sweden

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X-RAY microradiography, in conjunction with extraction and enzyme digestion, has been developed so that it can be used for quantitative determinations of substances of biological importance in single cells (2).

Fresh, frozen and dried sections of cells are exposed at 8 10 Å together with a reference system with the same wave length dependence, as shown in Fig. 1. After extraction with chloroform and digestion with ribonuclease, further exposures are made. The various fractions are obtained as differences in mass between the different exposures, expressed in $mg \cdot 10^{-9}/\mu^3$. The mass per unit surface of the reference system is assumed to be known, and the mass of the cell structure can thus be determined from the transmission values of the radiogram.

It is clear that the accuracy of the method is dependent on the quality of the reference system, and the accuracy with which its mass per μ^2 can be determined.

The reference system in Fig. 1 consists of 3 strips of a nitrocellulose film mounted in a step arrangement in slots cut in a metal segment. The film is prepared by spreading a nitrocellulose solution as a surface film on water. When the solvent has evaporated the film is removed from the water and allowed to dry. Part of the film is used for the reference system, and part is used to determine the mass per unit area, by weighing a known area of the film on a micro-balance.

Brattgård and Hallén (1) showed in 1951 that a nitrocellulose film 2–8 μ thick made without special precautions on a water surface shows variations in thickness, up to 70 per cent, even over quite small regions. Therefore these authors constructed an apparatus for the preparation of more uniform films. Their work shows that:

1. When a very uniform thickness is required over a large region of the

nitrocellulose film the apparatus becomes complicated and the method is time-consuming.

2. Even though the uniformity of the film is increased, variations in thickness are not completely eliminated. Since different parts of the film have to be used for determining the mass per unit area and for constructing the reference system, a factor of uncertainty is always involved.

3. In order to get a reasonably accurate average value one has to weigh a large number of known areas on the micro-balance. This is extremely time-consuming.

In a preliminary experiment Ingelstam and Hallén (3) showed that these difficulties can be avoided by measuring interferometrically the film thicknesses of the completed reference system. Knowing the specific weight of dry nitrocellulose the mass per unit area can then easily be found. In principle two different methods may be employed, one using multiple interferometry, the other two-slit interferometry.

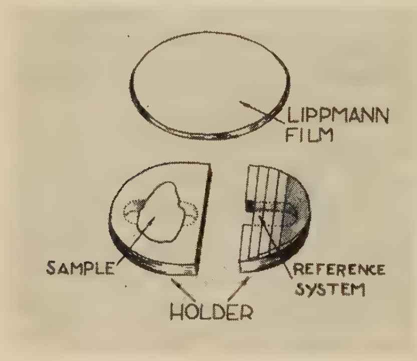

Fig. 1. Holder for sample and reference system.

The multiple interference measurements may be carried out easily with the Multimi interference microscope, developed by the Optics Laboratory and manufactured by AB C. E. Johansson, Eskilstuna.[1] Since only extremely small portions of the film are used in microradiography, this is of great importance when making an accurate survey of the thickness of the film. For routine work, however, this high accuracy is not necessary. The measurements are therefore made with a two-slit interferometer, giving an accuracy of a few per cent of the thickness of the film, which is quite sufficient.

The authors have developed this method, and constructed an interferometer which despite its simplicity allows rapid and reliable measurements of the step pattern in the reference system.

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[1] For further details and discussion of different possibilities see pamphlets from this company.

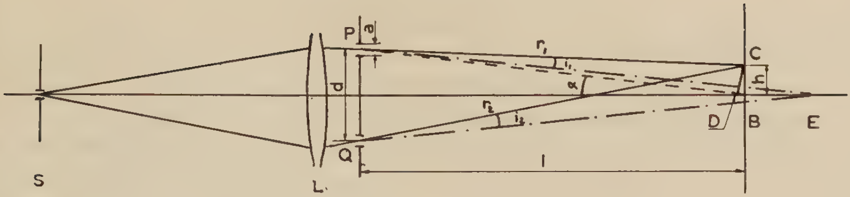

Fig. 2. Optical arrangements. Notations see the text.

Principle of the instrument

The apparatus is based on the classical interference experiments of Thomas Young in 1802. The principle is shown in Fig. 2. The entrance slit S is illuminated with monochromatic light, and for the treatment it can be assumed that the light from it is coherent, i.e. the phase is uniform over the narrow slit. The light from S falls on two subsequent slits P and Q, which function as two coherent light sources a short distance apart. Constructive or destructive interference fringes are formed on the screen B, depending on the distances which the two rays have travelled to reach the point in question. If the rays are in phase the intensity is increased, whereas they cancel each other if they are in antiphase and have the same amplitude. Between these extremes there are many intermediate cases, and the result is thus a continuously varying system of light and dark interference fringes. This will be considered in some detail, since the dimensions of the system must be chosen to suit the optical properties of the objects to be examined.

The rays from the slits P and Q are coherent, i.e. they are in phase with each other, and they may thus be represented $a_1 \sin 2\pi\ (t/T-\varphi)$ and $a_2 \sin 2\pi\ (t/T-\varphi)$, where φ is a phase angle, which is arbitrary and may be taken as zero.

The lens L images the entrance slit S at E. The width a of the slits is very small compared to l, and the wave front filling each slit may therefore be taken to be plane. According to the usual theory for the diffraction of light at a slit (4), the amplitudes in the directions i_1 and i_2, provided that the angles are small, are then $a_1 = a_{10}\ (\sin \pi a i_1/\lambda)/(\pi a i_1/\lambda)$ respectively $a_2 = a_{20}\ (\sin \pi a i_2/\lambda)/(\pi a i_2/\lambda)$.

The light wave at point C is formed by the superposition of waves from P and Q. The instantaneous value M of the amplitude is given by $M = a_1 \sin 2\pi(t/T-r_1/\lambda) + a_2 \sin 2\pi(t/T-r_2/\lambda)$, where r_1 and r_2 are the path lengths as shown in Fig. 2, and λ the wave length. If we set $2\pi r_1/\lambda = \delta_1$ and $2\pi r_2/\lambda = \delta_2$, we get $M = a_1 \sin\ (2\pi t/T-\delta_1) + a_2 \sin\ (2\pi t/T-\delta_2)$, which

may be written $M = A \sin(2\pi t/T - \beta)$ I
where $A^2 = a_1^2 + a_2^2 + 2a_1a_2\cos(\delta_2 - \delta_1)$ II
and β a phase angle.

For the present problem we are only interested in the intensity of the light, which is proportional to M^2, i.e. to A^2. It follows from the preceding equations that $\delta_2 - \delta_1 = (r_2 - r_1)\,2\pi/\lambda$. From Fig. 2 it is clear that, if BC is small, PB — PC $= h \sin\alpha$, where BC $= h$, and QC — QB $= h \sin\alpha$; so QC — PC $= r_2 - r_1 = 2h \sin\alpha = hd/l$, where d is the spacing between the centres of the double slits. The intensity in the image plane is thus given by $A^2 = a_1^2 + a_2^2 + 2a_1a_2\cos 2hd/l$. If the lens L focuses the rays at B, i.e. if B and E coincide, i_1 and i_2 are equal since $h \ll l$. If moreover there is no absorption in any of the slits, $a_{10} = a_{20} = a_0$ and thus

$A^2 = 4a_0^2\,[(\sin \pi a i/\lambda)/(\pi a i/\lambda)]^2 \cos^2 \pi h d/\lambda l$ . . . . II a.

The intensity vanishes if $\pi a i/\lambda = m\pi$ or $\pi h d/\lambda l = (k + {}^1/_2)\,\pi$ where m and k are integers. From Fig. 2 we get $i \approx h/l$. The intensity thus has minima at $h = ml\lambda/a$ and $h = (k + {}^1/_2)\,l\lambda/d$.

Now $d > a$, and thus the second factor in II a varies more rapidly than the first. We therefore get the intensity distributions shown in Fig. 3 and Fig. 4, the envelope being given by the first factor, which depends only on the slit width a.

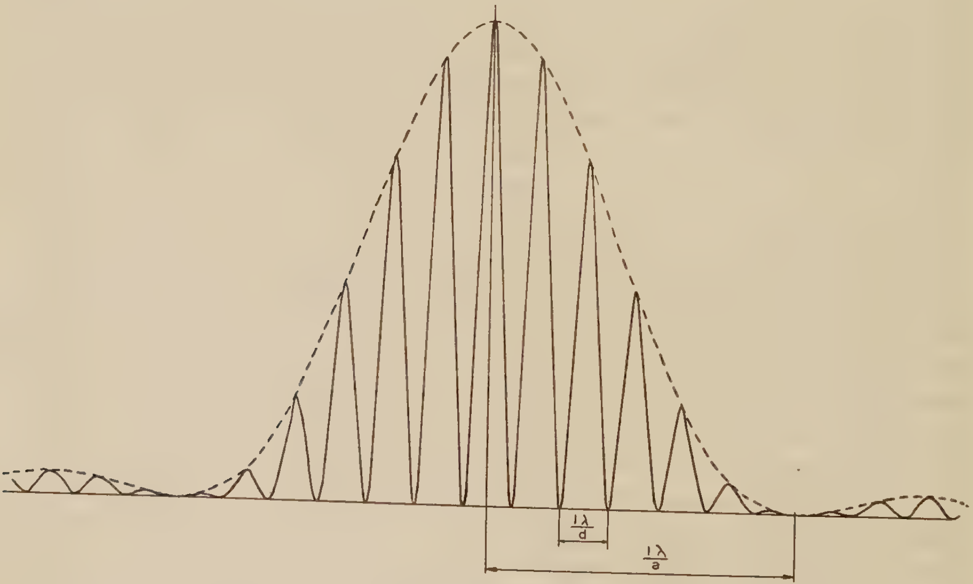

Fig. 3. The interference system.

If the slit source is not monochromatic the fringes are coloured. Since the interference system due to red light is more extended than that due to blue, the fringes are symmetric in colour about the central fringe (that for which $r_2 = r_1$). The fringes have blue edges towards the central fringe. At a certain distance from the centre the colours overlap to such an extent that the fringes become indistinguishable.

The above mentioned colour symmetry is used for measurements of the film thickness.

The reference system films are placed in the path of one of the rays (for example PC). The optical paths to the point at the distance h from the axis of the instrument about which the fringes are symmetric are then the same for both the slits. This gives $r_2 - (r_1 + (n-1)\,e) = 0$, where n is the refractive index, and e the film thickness. The geometrical path difference is $r_2 - r_1 = h\,d/l$ and thus

$$e = h\,d/l\,(n-1) \qquad \text{III}$$

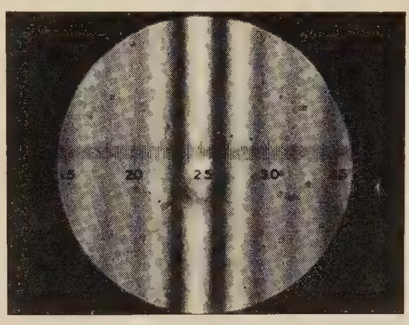

Fig. 4. The interference system photographed through the microscope.

Influence of loss of light by absorption and reflection in the sample

Equation II represents the cosine formula for the triangle shown in Fig. 5, which may conveniently be used to investigate the effect of loss of light. When h increases, a_2 rotates. The first minimum occurs when $\varphi = 180°$. If $a_1 \neq a_2$, i.e. if there is loss of light in one ray, then $A > 0$, and the minimum amplitude is greater than zero and the maximum amplitude is reduced. A loss of light of for example 15 per cent gives a ratio of minimum to maximum intensity of 1 : 150, and thus does not seriously reduce the visibility of the fringes. The loss in the cellulose films is certainly less than 15 per cent, and is therefore of no importance.

The central fringe, the position of which is measured, is displaced from the centre. Its intensity is also diminished according to the broken line in Fig. 3. For a large displacement the fringe may fall near the minimum at $l\lambda/a$ from the axis of the instrument. The envelope of the intensity curve is, however, only dependent on the width of the double slit. If this is reduced, the envelope curve spreads out, and the light distribution becomes more uniform, but the maximum intensity is reduced. A compromise must therefore be made between the intensity for maximum film thickness to be measured and the total intensity. This is done by giving the slit width a suitable value for each particular case.

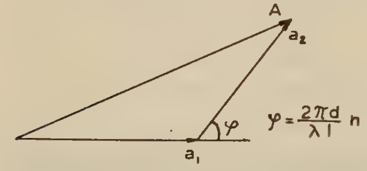

Fig. 5. Vector diagram for studying the influence of absorption and loss of light.

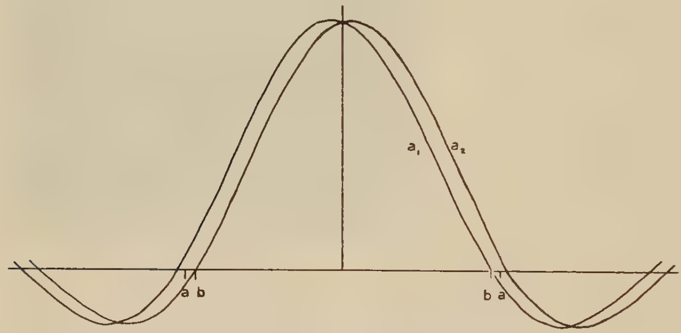

Fig. 6. Light amplitude vector distribution at unfocusing with lens L.

Influence of the slit width (*a*) *and of the focusing of lens L*

It was assumed above that the angles i_1 and i_2 (Fig. 2) were equal. If the lens L is adjusted so that the entrance slit is focused at E, i_1 and i_2 are unequal. The envelope of the light vector from P respectively Q are then displaced symmetrically according to Fig. 6. The region a–a, within which measurements can be made when the entrance slit is focused at B, is diminished to b–b. A distance BE of $l/5$ reduces here the distance a–a a few per cent. The light intensity distribution is a little changed as the amplitudes a_1 and a_2 are different. The defocusing is of no influence when it is small (BE less than $l/5$).

The effect of dispersion

The displacement of the central fringe is given as a function of n and e by equation III. If there is no dispersion in the film material (i.e. if n is the same for all wave lengths), the central fringe has the same colour as the light source, since the fringe systems of all wavelengths are displaced to the same extent from the original position. If, as is usually the case, n is lower for the F-line (4861 Å) than for the C-line (6563 Å), the displacement of the central maximum due to the cellulose film is less for the F-line than for the C-line, and the fringe becomes blue inside and red outside. It is then difficult to decide the position of the "centre of gravity" of the white light fringe. If the displacement between the two fringe systems at the central maximum is x, expressed as a fraction of the fringe width, $x = \Delta n \cdot e/\lambda$.

The value of x must be found experimentally. If for example $x = \frac{1}{4}$, $\Delta n = 0{,}011$, measured for cellulose films, and the average wave length 0,57 micron, the formula indicates that we can measure a cellulose film of greatest thickness $e = 13$ micron. Each outer fringe system is displaced with respect to the central system by half the above calculated amount. If it is desired to measure larger e values, the wave length range must be limited, so that the condition for x is satisfied. This can conveniently be done by using monochromatic light, or light with only a few lines, such as Hg-light. The central fringe is then located using unfiltered light, but for the exact measurement all lines except one are removed by filters.

The entrance slit

In the above calculation, which led to the intensity distribution shown in Fig. 3, it was assumed that the entrance slit was infinitely narrow. A finite slit may be represented mathematically by an infinite number of closely adjacent non-coherent infinitely narrow slits. That in the middle of the slit gives an intensity distribution as shown in Fig. 3, symmetrical about $h = 0$. The outer slits give similar distributions, but the line of symmetry is displaced along the h-axis by an amount corresponding to the displacement in S. The light intensity for different h-values in the image plane is obtained as the sum of the light from all sections of S. If the displacement of the symmetry line for the outer parts of the slit S is so great that it coincides with the minimum from the central zone of the slit, the fringes get diminished contrast and can become invisible. The finite width of the entrance slit always gives a reduction of the contrast. The slit width should be chosen so that it corresponds to less than the fringe half width value.

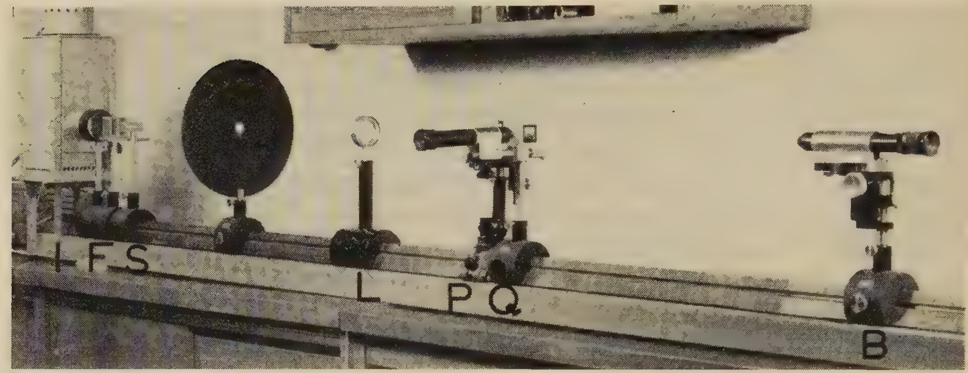

Fig. 7. View of built apparatus.

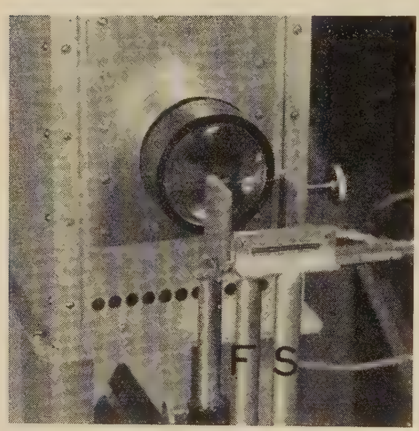

Fig. 8.

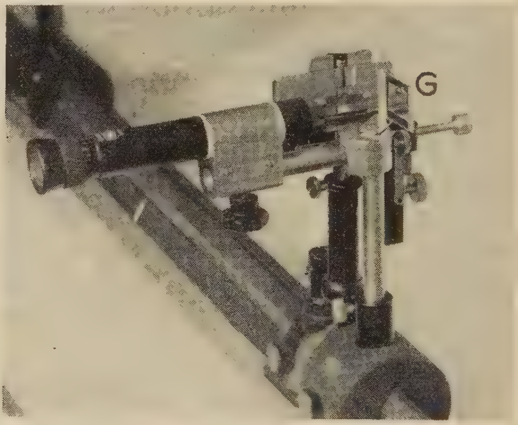

Fig. 9.

Fig. 8. Lamp housing, condenser, filter and entrance slit.
Fig. 9. Double slit, sample holder and microscope for adjustment of the reference system.

Description of practical apparatus

An apparatus based on the above theoretical principles has been constructed for the calibration of cellulose film reference systems. The assembly is shown in Figs. 7–9. The component parts are mounted on ordinary optical triangular prisms. Light from lamp I is focused by a condenser onto the entrance slit S. Between the condenser and the slit there is a filter holder. After the slit there is a screen to prevent any light from the lamp, which may pass outside the slit by scattering from reaching the observer at the microscope B. Fig. 9 shows the double slit and adjustable slide on which the reference system is mounted. By swinging the mirror G into the path of the rays the system can be examined by means of the microscope.

Several types of lamp have been tried, including "Pointo-lite", tungsten ribbon, and a high pressure mercury lamp (Osram HBO 200). The slits are narrow (see below) and the fringes will thus be weak unless a lamp of high luminance is selected. The mercury lamp has been used because it has the highest luminance of those tried.

The power of lens L is two dioptres, and the distance l is thus about 100 cm. Reference systems with e up to 10 micron can be measured (1). The double slit has been dimensioned so that a displacement for the central fringe to position b, Fig. 6, is equivalent to an e of about 16 microns, in order to make the central fringe situated in the region b–b of Fig. 3, and not too near position b, where the intensity and thus the accuracy of measurement are low. Convenient values for e and a are found to be 1500 and 130 microns respectively. The distance to b is then 4,2 mm. The fringe width is 290 microns for blue light (4300 Å) and for red light (6560 Å) 430 microns. As shown in the preceding section, the entrance slit should be considerably smaller than the average fringe width, and it has thus been set at 130 microns. Both the entrance slit and the double slit may conveniently be prepared by making a fine scratch in an aluminium layer evaporated on glass plate. The defocusing of lens L is not critical in this case. The extent of the region used is $^2/_3$ of the distance to the minimum at position b. As the defocusing in this case is slight, this is not disadvantageous.

When a measurement is to be made, the reference system is mounted in the holder, and adjusted with the help of the microscope, so that neither of the slits is covered by the layer. The microscope B is then adjusted so that the central maximum lies at the beginning of the scale (see Fig. 4). The various steps of the reference system are then placed in turn in front of one of the double slits, again using the microscope. The displacement of the central maximum is measured on the scale in microscope B. The thickness in mass per unit area of each step is then read off directly from a nomogram.

Table I shows a series of measurements and the accuracy which can be achieved. The displacement can be measured to ± 0.01 mm, corresponding to $^1/_{35}$ of a fringe. With the dimensions chosen for the apparatus this corresponds to an absolute value of ± 0.028 microns, or approximately 1 per cent of the thinner films.

The measurement gives a mean value for the thickness over the region through which the ray passes i.e. the area of the slit P. The reference systems were demounted and remounted between each measurement recorded in

Table I, since the three systems were examined in turn. The variation in the measured values are thus due to variations in thickness, as well as to observational errors, since the same part of each step has not necessarily been examined every time. For microradiographic applications the area used is smaller than that on which the measurement is made. The steps are constructed from specially selected films. Those films that show no interference colours when examined in light passing through them obliquely are assumed to be of uniform thickness. The accuracy given above thus includes any possible variations in *e*. The two-slit interferometer is not suitable for making a more detailed study of the topography of the film. If it is desired to make measurements of the same accuracy within smaller regions of the reference system an instrument such as the Multimi mentioned above should be used.

TABLE I

Reference system

Measurement Number	Displacement of the central fringe in mm		
	I	II	III
1	1.02–1.60–2.10	1.24–1.86–2.46	1.24–1.68–2.14
2	1.04–1.60–2.10	1.24–1.84–2.46	1.24–1.70–2.22
3	1.06–1.60–2.12	1.24–1.84–2.44	1.22–1.70–2.12
4	1.04–1.58–2.12	1.24–1.84–2.44	1.22–1.68–2.14
5	1.04–1.60–2.10	1.22–1.84–2.44	1.22–1.70–2.14
6	1.04–1.58–2.10	1.22–1.82–2.44	1.22–1.70–2.12
7	1.04–1.60–2.10	1.22–1.84–2.44	1.22–1.70–2.12
8	1.04–1.60–2.10	1.24–1.82–2.44	1.22–1.70–2.14
9	1.04–1.60–2.10	1.24–1.82–2.44	1.22–1.70–2.12
10	1.04–1.60–2.10	1.24–1.84–2.42	1.20–1.70–2.12

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MODIFICATION OF SOME ENZYMATIC CHARACTERS OF A STRAIN OF *STAPHYLOCOCCUS ALBUS* OBTAINED BY TREATMENT WITH DESOXYRIBONUCLEIC ACID FROM OTHER STAPHYLOCOCCI

M. U. DIANZANI

Department of General Pathology and Microbiology, University of Genova, Genova, Italy

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The study of bacterial mutations, induced by nucleic acids is a field of general interest. This was demonstrated by Griffith (16), Dawson and Sia (9), Alloway (2, 3), and Avery *et al.* (4), working with pneumococci. The type transforming principle was identified as a desoxyribonucleic acid. Analogous successful investigations were performed by Boivin *et al.* (5) on *Escherichia coli,* by Weil and Binder (26) on *Shigella paradysentheriae* and by Alexander and Leidy (1) on *Haemophilus influenzae.* In previously reported experiments (9) the present author also obtained in one strain of *E. coli* and in one strain of *Proteus* OX 19 the appearance of new, originally missing enzymatic characters, by treatment with desoxyribonucleic acid (DNA) isolated from bacteria of the same or of a very related species in which the enzymatic characters were present.

The possibility for the acquisition of new characters in staphylococci by treatment with substances of bacterial origin was studied by Byatt *et al.* (6), who describe the acquisition of specific pigment by a strain of *Staph. albus* after treatment with extract from another actively pigment-producing *Staph. albus* strain. It is, however, very uncertain if this phenomenon can be interpreted as a directed mutation, because the pigment production is an extremely variable character, occurring under a number of different experimental conditions.

A new study of the problem is presented in this paper, particularly with regard to the possibility of the acquisition of new enzymatic characters and to the role of DNA.

METHODS

A number of strains from our collection were used. As cultivation medium was used ordinary 3 per cent agar The oxidative activities were studied with Warburg technique.

The bacterial suspensions were prepared by removing with saline the surface growth from 24 hour agar cultures. The bacteria were sedimented in a centrifuge at 5000 rpm., washed 3–4 times with saline and finally suspended in 6.5 ml of a phosphate buffer (0.067 M, pH 7.4). 3 ml of this suspension was transferred to each of two Warburg vessels. 0.20 ml of the substrate was added to one of the vessels. 0.20 ml KOH (30 per cent) was placed in the central cups. The manometers were put in the constant temperature bath, kept at 38° C, and after 7–8 min. for temperature equilibration the oxygen consumption was followed for 60 minutes. The difference in O_2-uptake between the manometer containing substrate and the control one was calculated, and this difference expressed in per cent of the control value was used as a measure for the oxidative activities. The following substrates were used: 1) ethanol, glycerol, mannitol, d(+)-xylose, arabinose, glucose, galactose, levulose, maltose, sucrose, lactose, raffinose, salicine, dl-alanine, dl-leucine, dl-histidine hydrochloride, l(+)-arginine, dl-methionine, all in the final concentration of 0.0125 M. 2) dl-valine, dl-tryptophane, dl-cystine, 0.062 M. 3) dl-tyrosine, 0.000125 M. 4) soluble starch, dextrine and inuline in a conc. of 0.062 per cent.

At the end of each experiment the dry-weights of the bacterial suspensions were determined (after heating to 110° C until constant weight), and the QO_2 values (mm^3 O_2 per hour per mg dry weight) were calculated.

Strain used. Three strains, here designed as GT, FRA and TA, which in the preliminary experiments had shown interesting properties were particularly investigated.

1. Staphylococcus GT was white, not pigment producing, in R-form and rapidly agglutinable by trypaflavine. It was nonhemolytic and a weak coagulase-producer. Clearly demonstrable capsule was missing, but a very thin layer of capsular material could be demonstrated by staining according to Hiss. It was avirulent for the rabbit (tested by intravenous injection of 2 ml of a 24 hours broth culture).

2. Staphyloccus FRA was actively pigment producing, in S-form with distinct capsule, a good hemolysin- and coagulase-producer and highly virulent for the rabbit (2 ml of a 24 h. broth culture killed the animals within 24 hours).

3. Staphylococcus TA was a weak pigment producer, agglutinable by trypaflavine, a good coagulase producer and a weak hemolysin producer, with a very thin capsule. Intravenous injection of 2 ml of a 24 h. broth culture killed the rabbits within 5 days. Staphylococcus TA in pure culture was isolated from abscessus-material from the animals.

EXPERIMENTAL DATA

The strains GT, FRA and TA showed greatest difference with respect to the capacity of oxidizing amino acids. Particularly pronounced difference was found in the oxidation of methionine, which substrate was not attacked by GT but oxidized by FRA and TA, and of cystine, which was hardly oxidized by GT, but easily attacked by FRA and TA. The oxidative activities of the mentioned strains are summarized in Table I. As a rule the given figures represent the mean values of 4 determinations.

TABLE I

Oxidations of various substrates by the strains GT, FRA and TA. (The data are expressed as percentages of increase of O_2-uptake which occurred in the presence of the substrate. — Manometric technique of Warburg. Temperature: 38° C.)

Substrate	Strains GT	 FRA	 TA
arabinose	+ 72.48	+ 12.10	+ 20.60
d(+)-xylose	+ 55.16	+ 11.28	+ 42.12
glucose	+ 34.35	+ 441.64	+ 80.70
levulose	+ 361.32	+ 411.74	+ 68.14
galactose	+ 15.16	+ 16.28	+ 17.77
maltose	+ 31.71	+ 50.03	+ 40.60
sucrose	+ 195.06	+ 190.14	+ 96.44
lactose	+ 91.01	+ 21.09	+ 53.15
raffinose	+ 54.29	+ 58.7	+ 52.60
dextrine	+ 81.77	+ 74.13	+ 16.18
soluble starch	+ 11.8	+ 12.45	+ 6.44
inuline	+ 109.92	+ 146.33	+ 12.38
salicine	+ 5.66	− 0.58	− 2.26
ethanol	+ 69.9	+ 87.9	+ 96.15
glycerol	+ 319.65	+ 453.33	+ 278.10
mannitol	+ 21.39	+ 91.9	+ 40.12
dl-alanine	+ 104.08	+ 189.73	+ 60.71
dl-valine	− 20.26	− 19.62	+ 3.35
dl-leucine	+ 30.38	+ 36.0	+ 42.45
dl-histidine	+ 24.95	+ 121.19	+ 26.12
l(+)-arginine	+ 129.96	+ 12.16	+ 46.13
dl-tryptophane	+ 29.5	+ 10.26	+ 9.21
dl-tyrosine	+ 25.25	+ 71.64	+ 16.28
dl-methionine	− 5.55	+ 29.08	+ 28.21
dl-cystine	+ 1.10	+ 23.51	+ 29,41

The data for the strain GT are in fairly good agreement, also quantitatively, with those reported by Pasquinelli and Lamanna (21) who used the same strain for an investigation of the oxidative activities of some staphylococci against amino acids. They reported lack of methionine-oxidation. The cystine-oxidation was not investigated by those authors, but they observed a considerable increase of oxygen consumption in the presence of cysteine. As the values of ammonia production are much lower than the theoretically expected ones, only a small fraction of the increase of oxygen consumption can be referred to substrate oxidation. The explanation for the increase which is given by Pasquinelli and Lamanna (21), namely that cysteine stimulates the oxidation of other substrates present in the bacteria, is the one suggested by Califano (7) and Califano and Ciaranfi (8). More probable is, however, that the effect is due to the spontaneous auto-oxidation of cysteine, which is known to take place at a very high rate. This last interpretation is supported by the fact, established by the present investigation, that the strain GT does not attack cystine, the oxidized derivative of cysteine.

The following part of the investigation was undertaken in order to ascertain, if the treatment *in vivo* of the deficient strain GT with dead bacteria of the strains FRA and TA, or *in vitro* with DNA isolated from those strains would induce any new stable enzymatic characters in GT.

Experiments in vivo. One ml of a 24 hours broth culture of the strain GT was mixed with 1 ml of similar cultures of FRA and TA, previously heat-killed. These mixtures were injected intravenously in rabbits. Animals were also injected with the heat-killed suspensions of FRA and TA only, and others with the original GT. The rabbit inoculated with the mixture of GT and heat-killed FRA died within 36 hours. A white staphylococcus, designed as C_1, was isolated from blood, liver, heart, spleen, kidney and bone-marrow. It was in R-form, provided with a very thin capsule and very similar to the original GT in cultural characteristics. The oxidative activities of the strain C_1 are summarized in Table II. Intravenous injection of 2 ml of a 24 hours broth culture of C_1 in rabbits caused no symptoms of illness within an observation period of two months.

The rabbit, inoculated with 1 ml of heat-killed TA plus 1 ml of GT, died 10 days after injection. Two large abscesses in the ileo-psoas region and one smaller at the cardiac apex were found at the autopsy. A white staphylococcus, designed as C_2, was isolated from the abscess-material. It was in R-form, had a very thin capsule and was very similar to GT in cultural characters. The oxidative activities of this strain are also reported in Table II. C_2 was avirulent for rabbit.

Rabbits injected with heat-killed suspensions of FRA and TA were completely unaffected. The strain GT was avirulent. One rabbit injected with 2 ml of a 24 hours broth culture of GT was killed by bleeding 24 hours after the injection. A white staphylococcus, designed as C_3, was isolated from the blood. It was in R-form, with a thin capsule and with cultural characteristics very similar to those of GT. It was avirulent for rabbit. The oxidative activities of C_3 are found in Table II. C_3 seems to be almost identical with GT with regard to biochemical activities. C_1 and C_2 show some differences by comparison with GT. C_1 oxidizes methionine and C_2 methionine and cystine. These substrates are oxidized by FRA and TA but not by GT. With regard to other substrates C_1 and C_2 are, in general, more related to GT than to FRA and TA. For instance, C_1 as well as GT strongly oxidize arabinose and d(+)-xylose, which are only weakly oxidized by FRA, and they also show a lower capacity of oxidizing dl-histidine than FRA does. C_2 and and GT oxidize arabinose, d(+)-xylose and tryptophane better than TA. After animal passage, however, the metabolic activities of C_1 and C_2 are considerably higher than those of the original

TABLE II

Oxidation of various substrates by the strains C_1, C_2 and C_3. (The data are expressed as percentages of increase of O_2-uptake which occurred in the presence of the substrate. Manometric technique of Warburg. Temperature 38° C.)

Substrate	Strains		
	C_1	C_2	C_3
arabinose	+ 68.32	+ 75.60	+ 70.40
d(+)-xylose	+ 50.14	+ 60.15	+ 50.78
glucose	+ 589.50	+ 180.16	+ 360.60
levulose	+ 371.94	+ 190.44	+ 380.40
galactose	+ 61.17	+ 27.38	+ 68.06
maltose	+ 271.91	+ 67.74	+ 60.96
sucrose	+ 200.60	+ 206.80	+ 200.48
lactose	+ 100.48	+ 112.60	+ 110.40
raffinose	+ 66.18	+ 60.40	+ 68.40
dextrine	+ 78.15	+ 86.12	+ 104.30
soluble starch	+ 10.60	+ 12.40	+ 10.20
inuline	+ 110.12	+ 60.40	+ 86.12
salicine	− 4.06	− 10.08	− 3.74
ethanol	+ 259.40	+ 160.40	+ 76.40
glycerol	+ 552.54	+ 440.60	+ 360.60
mannitol	+ 113.75	+ 82.40	+ 40.70
dl-alanine	+ 196.41	+ 120.23	+ 136.40
dl-valine	− 16.60	+ 0.92	− 7.30
dl-leucine	+ 33.33	+ 40.54	+ 36.40
dl-histidine	+ 57.52	+ 18.48	+ 30.60
l(+)-arginine	+ 144.67	+ 35.25	+ 140.70
dl-tryptophane	+ 36.15	+ 20.14	+ 36.40
dl-tyrosine	+ 97.95	+ 14.99	+ 20.40
dl-methionine	+ 36.36	+ 31.99	− 6.40
dl-cystine	− 1.68	+ 20.42	+ 1.46

strain GT. The characteristics of the strain C_3 are, apart from smaller quantitative differences, essentially identical with those of GT. The oxidative capacities acquired by C_1 and C_2 remained unchanged still 12 months after the isolation of the strains.

Experiments in vitro. These experiments were performed in order to study, if the above described modifications can be acquired after treatment *in vitro* with nucleoprotein (NP) isolated from FRA or TA. The technique of Vendrely (21) was used for isolation of NP.

GT was treated *in vitro* with NP in the following way: a small amount of the NP on a platinum loop was intimately mixed on the agar surface with a very small amount of GT growth, diluted in a drop of sterile physiological saline. The fundamental purpose was to bring a small number of cocci in contact with a large amount of NP. Daily passages were made in a similar way. The oxidative activities against methionine and cystine were measured at short intervals. No change in those activities could be detected even after 42 passages.

Alloway (3), Dawson and Sia (2) and Avery *et al.* (4) have shown that type-transformation of pneumococci will occur exclusively in the presence of anti-R-serum, physiological exudates or normal serum. Treatment with NP was therefore repeated in presence of normal inactivated horse serum. The same technique as previously described was used in these experiments, with a slight modification, namely that the bacterial growth was diluted in a drop of fresh normal horse serum instead of physiological saline. Control experiments with NP alone or with serum alone were performed parallelly. The treated strains were tested at short intervals of time for oxidative capacities against methionine and cystine.

After 5 passages with NP from FRA in presence of serum, the treated strain showed a small oxidation of methionine (increase of O_2-uptake in presence of methionine 15.3 per cent) and of cystine (increase 10.7 per cent). This capacity increased with continued passages to attain, after 10 passages, the values 35.4 per cent for methionine and 16.7 per cent for cystine. No further increase was observed in the following passages. Treatment with serum only, i.e. without NP, could not induce any modification of the oxidation of methionine even after 34 passages. Cystine produced only a very moderate increase in the oxygen-consumption (4.6 per cent), which did not rise further during the continued passages, and disappeared shortly after the treatment was discontinued.

The capacity to oxidize methionine and cystine, induced in GT by serum plus NP from FRA, is now remaining unchanged even after 10 months.

In another experiment the same strain GT, after a similar treatment with NP from TA in the presence of normal horse serum, began to show oxidation of methionine (increase of O_2-uptake 25.4 per cent) and of cystine (increase 10.3 per cent) even after 5 passages. The oxidative activities increase during the further passages and the following values were obtained after 12 passages: 40.2 per cent for methionine, 22.7 per cent for cystine. The acquired oxidative activities were qualitatively unchanged after 10 months.

The whole procedure was repeated in the presence of normal horse serum and NP from FRA and TA, but previously treated with crystalline ribonuclease, prepared according to MacDonald (19), with purified desoxyribonuclease, prepared according to McCarty (8), and with trypsin. The modifications appeared in exactly the same way after digestion of the NP with ribonuclease or with trypsin, but not after treatment with purified desoxyribonuclease.

The strains which were obtained after 5 passages with NP from FRA and TA in the presence of horse serum, denoted GTsFRA 5 and GTsTA 5, and showed cultural characters very similar to those of the original strain GT. They were not pigment-producers, were inagglutinable by trypaflavine, had only a very thin capsule and were weak coagulase-producers. GTs FRA 5 differed from GT in being a weak hemolysin-producer. GTs FRA 5 and GTs TA 5 were avirulent for the rabbit.

No modification of the metabolic or cultural characteristics of GT were observed after treatment with NP isolated from the original strain GT or with material from another staphylococcus (DA), unable to oxidize methionine and cystine.

DISCUSSION

Are the phenomena, described in the present paper attributable to directed mutations, to processes of selection or to adaptation?

The experiments *in vivo* show that an avirulent strain mixed with a heat-killed, virulent strain of staphylococcus will cause death of the animals. The pathological alterations found are very similar to those typical for the inducing pathogenic strain. The strains isolated from the blood and from the organs of the dead animals, C_1 and C_2, had cultural and biochemical characters very similar to those of the original, avirulent strain, but differed with respect to the oxidation of methionine, acquired by both strains obtained, and of cystine, which was attacked by one of them. Methionine and cystine was easily oxidized by the inducer strains, but not by the original GT strain.

The fact that the strains C_1 and C_2 were unable to kill the rabbits, suggests that the death of the animals observed in the fundamental experiments is not conditioned by the mutative acquisition of a "virulence character", but must be ascribed to phenomena of a different nature. Some phenomena very similar to those reported here have been described for pneumococci. Rosenow (22) showed that virulent pneumococci contain a substance capable of inhibiting the phagocytosis of both virulent and avirulent pneumococci by granulocytes. In 1926 this substance was identified by Sia (23), as the specific capsular polysaccharide. In 1926 Felton and Bailey (13) showed that the inoculation of mice simultaneously with the specific polysaccharide from virulent pneumococci type II and an avirulent strain of the same type produces a fatal infection which cannot be established by the avirulent strain alone. Very recent investigations by Felton *et al.* (14), by MacLeod and Krauss (20), and by Wood and Smith (27) have confirmed that the virulence of pneumococci is connected with the antiphagocytotic activity of the specific polysaccharides which immobilizes granulocytes and then permits the multiplication of the microorganisms in a favourable medium. Investigations by Gilbert (15) indicate that the virulence of staphylococci is depending upon the presence of a capsular polysaccharide. A capsulated strain of *Staphylococcus aureus,* highly virulent for the guinea pig, became avirulent with the loss of capsule and recovered virulence with reappearing capsule. Confirming experiments are reported by Julianelle *et al.* (17). Verwey (25) will, however, attribute the virulence of staphylococci to a type-specific protein. The death of rabbits reported in this paper could probably be referred to an "immunological paralysis", provoked by substances from the killed, originally virulent strains.

It seems, however, more difficult to explain the biochemical changes observed in the strains isolated from the dead animals.

Out of the *in vivo* experiments no decision can be made as to whether directed mutations or selective processes prevail. The increase of already in the GT strain existing metabolic activities can be explained as an adaptation. The capacity of oxidizing cystine, found in the C_2 strain, is not completely missing in GT, but rather weak (1.1 per cent increase). C_1 did not, however, oxidize cystine, which could have been expected in the case of a simple adaptation.

The *in vitro* experiments show that treatment of the GT strain by nucleoprotein fraction from FRA and TA will induce the capacity of oxidizing methionine and cystine. There is no evidence for the presence in the nucleoprotein fraction of methionine and cystine in amounts large enough to cause an adaptation. The integrity of the DNA is an indispensable condition for the positive result of the modification experiments, as nucleoprotein, previously treated with desoxyribonuclease, had no effect. It is not very probable that the appearance of new, stable characters means a mobilization of latent characters by a more unspecific action of the nucleoproteins. The treatment with nucleoprotein from the same strain GT or from another DA, also unable to oxidize methionine or cystine, had no result; only the nucleoproteins isolated from FRA and TA were effective. The analogies in experimental conditions to the transformation of pneumococci favour the hypothesis of mutation without completely excluding a selective process.

SUMMARY

The appearance in a strain of staphylococci of new, stable oxidative activities against methionine and cystine is described. This is a consequence of the treatment of the strain *in vivo* with dead cells from two other strains having the mentioned activities, or *in vitro* with nucleoprotein fractions from those strains. Desoxyribonuclease destroys the inducing capacity of the nucleoproteins; ribonuclease and trypsin do not. The interpretation of the results is briefly discussed.

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THE SYNTHESIS OF NICOTINIC ACID BY *CHILOMONAS PARAMECIUM*

P. S. STOKELY

University of Notre Dame, Biology Department, Notre Dame, Indiana, U.S.A.

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By the use of a chemical method of assay, Hutchens, Jandorf and Hastings (2) showed that cells of *Chilomonas paramecium* contain nicotinic acid after having grown in a medium free of this vitamin. It appears that more precise information concerning the synthesis of nicotinic acid might be obtained by the use of a microbiological assay.

A bacteria-free clone of *Chilomonas paramecium* was cultured in a medium which was essentially that of Hutchens *et al.* (2) but modified slightly with regard to iron and thiamin content. (The medium of Hutchens *et al.* contained 1.70 mg $FeCl_3 \cdot 6H_2O$ and 0.10 mg thiamin hydrochloride per liter of solution compared with the present author's use of 4.064 mg $FeCl_3 \cdot 6H_2O$ and 0.160 mg thiamin hydrochloride per liter of solution.) After adjusting the pH to 6.6 the medium was dispensed in 50 ml volumes into 125 ml Erlenmeyer flasks and autoclaved. After inoculating with the chilomonads the flasks were incubated at 26.5° C in a water bath. After 48 hours the cells were amassed by slow centrifugation into previously weighed shell vials and dried to constant weight. Approximately 4 mg of dried cells were obtained after 48 hours from each 50 ml of culture fluid.

Three general types of extraction of the nicotinic acid from the dried cells were employed: digestion with enzymes; extraction by hot water; acid hydrolysis. Where enzymatic digestion was employed, the procedure was that suggested by Cheldelin, Eppright, Snell and Guirard (1). The extractions made by hot water were accomplished by adding 10 ml of distilled water to the dried cells and autoclaving at 11 lbs. for 30 minutes. Upon cooling the contents were diluted up to 15 ml and centrifuged, the clear supernatant liquid being taken as the extract. For acid hydrolysis, 2.0 ml of 1.5 N HCl was added to the desiccated cells and the vial and its contents autoclaved for 30 minutes at 11 lbs. The pH was then adjusted with NaOH to approximate that of the basal medium into which it would be later introduced for assay. This procedure was followed by dilution with distilled water up to 15 ml and centrifugation to secure a clear extract.

The microbiological method of essay for nicotinic acid used was the one presented by Snell and Wright (6) employing a nicotinic acid free basal medium and *Lactobacillus arabinosus* 17-5 as the test organism. Standard dilutions of 0.00, 0.05, 0.10, 0.20, 0.30, 0.50, 0.70, and 1.00 gamma of nicotinic acid (Smaco) respectively were used to bring the 9 ml of basal medium in each bacteriological test tube up to 10 ml. These were then plugged with cotton and autoclaved at 11 lbs. for 25 minutes.

Stock cultures of *L. arabinosus* were carried in yeast extract glucose agar. For use in the assays, cells were transferred into a tube of basal medium containing sufficient nicotinic acid to support growth (0.1 gamma/10 ml), and after 24 hours at 30° C the bacteria were centrifuged out under aseptic conditions and resuspended in 10 ml of 0.9 per cent saline solution. One drop of the resulting suspension was added to each assay tube and the entire lot was then incubated at 30° C for 72 hours.

Titration of the lactic acid produced during growth was used as a measure of the response to nicotinic acid, there being, within definite limits, a direct proportion between the amounts of nicotinic acid present and the volumes of lactic acid produced as a function of the growth of the test organism. Titrations were made with approximately 0.1 N NaOH, with phenolphthalein as the indicator. The bacterial cells were thrown out of suspension by centrifugation and from each tube 5 ml of the supernatant liquid was taken. The volume of base necessary to neutralize this volume of fluid was noted. It was possible then to construct a standard curve, the concentration of nicotinic acid in the standard, known tubes being the abscissae and the volumes of NaOH necessary to neutralize the lactic acid present being the ordinates. A typical standard curve is shown as Fig. 1.

It was found that the use of 0.25, 0.50, 0.75, and 1.00 ml portions of the 15 ml of extract were suited for assay and gave results distributed along the sensitive portion of the standard curve. Where ever necessary, the aliquot chosen was diluted up to 1 ml by adding distilled water. The nicotinic acid content of an aliquot of extract was determined in this manner; the amount of nicotinic acid in any portion was considered the abscissa intersecting the standard curve with the ordinate which was the amount of NaOH necessary to neutralize the amount of lactic acid present in the portion. This particular amount was then multiplied by its dilution factor (e.g., 0.25 ml by 4). The average of the amounts so ascertained from the four different dilutions of a single extract were considered a rather accurate estimate of the amount of nicotinic acid present in the extract.

Recovery of known amounts of nicotinic acid by the dilution method was used a check on the reliability of this type of assay. Table I presents the results of five recovery runs, each made with a different standard curve; the average recovery being 99 per cent. Examination of the table shows, however, that the variations above and below 100 per cent recovery extend over a range of 16 per cent. This indicated the necessity of a check on each run; and, therefore, runs became routine procedure with each assay. The results of each assay were corrected by the amount indicated by the percentage recovery of known amounts of nicotinic acid ascertained on that standard curve.

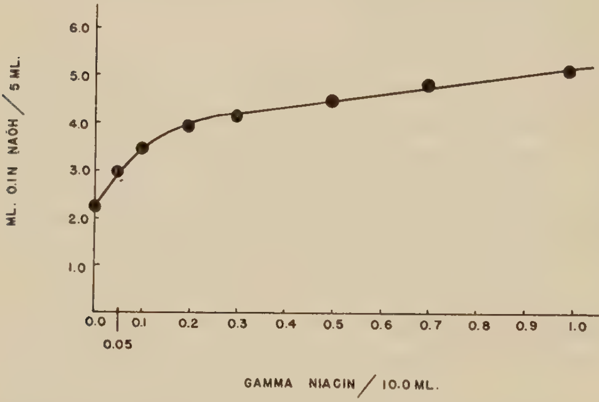

Fig. 1. A typical standard curve showing the relations between the volume of 0.1 N NaOH necessary to neutralize the lactic acid present in 5 ml of fluid in which *Lactobacillus arabinosus* had been growing for 72 hours in the presence of known amounts of nicotinic acid.

The relative efficacy of the various methods of extraction is indicated by the mean values and their standard errors of the four kinds of experiments as follows:

Takadiastase	0.123 ± 0.0054 γ/mg
Takadiastase and papain	0.119 ± 0.0118 γ/mg
Hot water	0.148 ± 0.0147 γ/mg
Hot hydrochloric acid	0.124 ± 0.0110 γ/mg

Attempted extraction with papain alone resulted in completely negative results. Inspection of the above list shows that three of the figures are not significantly different from one another. The difference between the two most different readings (takadiastase and papain compared with hot water) is only 1.6 times the standard error of the differences (29/18.8), indicating that statistically all the methods of extraction (papain excepted) yield the same result. Extraction with hot water tended to give higher results more frequently and, therefore, it is conceivable that, when many more hot water extractions and assays of them are made, the error may be cut low enough

TABLE I

Summary of results of assays for nicotinic acid content expressed in gamma/mg dry weight of *Chilomonas paramecium*. "Grand average" omits the result obtained after papain extraction.

Method of extract.	Exp. No.	Dry wt. animals (mg)	Nicotinic acid found (γ)	Nicotinic acid content (γ/mg)	% nic. acid rec. from known	Corrected nic. acid con. (γ/mg)	Average each extraction (γ/mg)	Grand Ave. (γ/mg) ±S.E.
Takadiastase (24 hrs.)	927 a	59.5	6.069	0.102	95.9	0.106	0.123	0.129 ±0.0058
	928	55.8	7.560	0.135	100.7	0.133		
	928 r	55.8	6.640	0.119	98.7[1]	0.120		
	101	49.2	6.480	0.132	100.7	0.131		
Papain (24 hrs.)	914	313.2[2]	1.815	0.005	93.5	0.006	0.007	
	918	68.1	0.610	0.009	103.0	0.008		
Tak. and papain (24 hrs.)	927 d	58.5	5.089	0.087	95.9	0.090	0.119	
	930 a	32.5	4.485	0.138	100.7	0.136		
	930 b	35.7	4.710	0.132	100.7	0.131		
Hot water	927 b	57.8	6.185	0.107	95.9	0.111	0.148	
	930 d	32.0	5.205	0.163	100.7	0.161		
	105 b	48.5	8.342	0.172	98.7[1]	0.173		
Hot HCl	930 c	35.2	4.470	0.127	100.7	0.125	0.124	
	930 cr	35.2	3.696	0.105	98.7[1]	0.106		
	105 a	50.9	7.710	0.151	98.7[1]	0.152		

to yield a figure significantly different from the others. On the basis of the evidence on hand, however, it appears that nicotinic acid is freed from the dried cells of *Chilomonas paramecium* in equal amounts when takadiastase, takadiastase and papain, hot water, and hot hydrochloric acid are used as the extracting agents.

The conclusion is drawn that the grand average of the four results — 0.129 ± 0.0058 γ/mg dry weight (mean ± S.E.) — represents a fair statement of the amount of nicotinic acid present in *C. paramecium* grown under the conditions stated above in this report. That the nicotinic acid present in the cells is synthesized by the cells themselves is indicated by the fact that the only two possible extraneous sources yielded no significant activity when assayed: 1) Takadiastase suspension (20 mg — 30 times the amount added in the extracts) treated in the manner in which the extracts were treated during digestion showed no growth activity; and 2) 5 ml of culture fluid in which animals had grown for 72 hours also showed no activity.

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[1] three values used to obtain average; [2] wet weight; "*r*" indicates repetition of assay using formerly tested extract to test reproducibility.

The microbiological method of assay is not highly specific since it is impossible to ascertain what fraction of the results obtained indicates free nicotinic acid, what fraction represents cozymase, and what fraction may represent the growth promoting activities of compounds which may substitute for nicotinic acid or nicotinamide. If we assume that all of the nicotinic acid present in the chilomonads as indicated by the results of the assays recorded here is bound in the diphosphopyridine nucleotide (cozymase) molecule, then we can expect an assay for the cozymase to yield 0.750 γ/mg of dryweight of *C. paramecium.* Snell and Wright (6) found nicotinic acid to represent 17 per cent of the cozymase. According to the findings of Hutchens *et al.* (2), *C. paramecium* contains 0.85 ± 0.06 gamma of diphosphopyridine nucleotide per mg of dry cells. This estimation was made by using the manometric method described by Jandorf, Klemperer and Hastings (3). Since nicotinic acid and its amide do not respond to this test, the figure 0.85 represents only the cozymase present in the organism ((5), p. 234). Comparison, then, of the amount of cozymase represented by the nicotinic acid found in this present investigation with the amount of cozymase found by Hutchens *et al.* reveals a difference of only 11.7 per cent. In other words, if it is assumed that 0.85 γ/mg of dry cells does represent the true amount of diphosphopyridine nucleotide present in the organism and that 17 per cent of this amount is nicotinic acid, then these experiments reported on here show the recovery of 88 per cent of the amount found by Hutchens. Since the assays of both these compounds are variable to some extent (according to Leonian and Lilly (4) a 10 per cent variation in assay figures is considered very good, and 15 or even 20 per cent permissible in some cases), the apparent difference may not be real at all, and the results may indicate perfect agreement. If this is true then the nicotinic acid is present in the cells of *C. paramecium* only in the form of diphosphopyridine nucleotide.

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THE EFFECT OF TRYPSIN ON THE CROSS FERTILIZABILITY OF SEA URCHIN EGGS

A. BOHUS JENSEN

The Bermuda Biological Station, The Bermuda Islands; The Institute for Cancer Research and the Lankenau Hospital Research Institute, Philadelphia, Pa., U. S. A., and The Biological Institute of the Carlsberg Foundation, Copenhagen, Denmark[1]

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In certain combinations of sea urchin species only a low percentage or none of the eggs are fertilizable by sperm from the other species shortly after the removal of the eggs from the gonads. The eggs can be made cross fertilizable, however, by various means, but very little is at present understood about the manner in which these treatments affect the eggs (2, 4, 6, 7, 8, 9, 10).

Hultin (7, 8) has recently shown that a treatment of sea urchin eggs with an impure trypsin preparation (Kahlbaum) makes them cross fertilizable. In view of the previous finding that the fertilization of sea urchin eggs is inhibited in a reversible manner by crystalline trypsin (1), it became of interest to find out whether a crystalline trypsin preparation would affect the eggs in the way described by Hultin. With that purpose in mind the present study was made on a different kind of sea urchin material.

MATERIALS AND METHODS

The investigation described in this paper was carried out at the Bermuda Biological Station for Research[2] during July–Sept. 1952 with egg and sperm material from the following species of sea urchins: *Lytechinus variegatus* (Lamarck), *Mellita sexiesperforata* (Leske) and *Echinometra lucunter* (Linnæus). In each experiment a batch of eggs from a single female was used and the quality of the egg material was tested before the experiment by establishing the percentage of fertilizable eggs. The eggs were washed in sea water before use and no attempts were made to remove their jelly coats. The criterion used for a successful cross fertilization was a normal or fairly normal cleavage to about blastula stage. The experiments were carried out at room temperature (28–30° C). The crystalline trypsin was obtained from Armour Laboratories, Chicago and Worthington Biochemical Sales Co., Freehold, N.J. USA. The trypsin solutions were dialysed before use against normal sea water for 24 hours in a refrigerator. The concentration of trypsin given in the text includes the inorganic salts contained in the original enzyme preparations but afterwards removed by dialysis.

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[1] present address.

[2] Contribution from the Bermuda Biological Station for Research no 199.

EXPERIMENTS

No information is apparently given in the literature about the cross fertilizability of *Lytechinus* eggs by spermatozoa from *Mellita sexiesperforata* and *Echinometra lucunter.* Tennent (9), however, says without giving further data that crosses were made between *Lytechinus* eggs and spermatozoa from *Mellita quinquiesperforata* (Leske) after the eggs had stood in sea water for 5 hours. Two series of orienting experiments were therefore performed with eggs from *Lytechinus* and spermatozoa from *Mellita sexiesperforata* and *Echinometra lucunter.* They may be summarized as follows: In 5 experiments *Lytechinus* eggs were inseminated by *Mellita* spermatozoa with the result that 0–2.4 per cent of the eggs showed cleavage. In 7 experiments *Lytechinus* eggs were inseminated with *Echinometra* spermatozoa and subsequently *1.1–9.2* per cent of the eggs went into cleavage. In the 12 control experiments where *Lytechinus* eggs were inseminated with *Lytechinus* spermatozoa, *76.3–100 per cent* of the eggs began cleavage. The conditions in experiment and control may not be strictly comparable, since the procedure did not ensure that the same concentration of sperm cells was present in the experimental and control samples. *Egg samples from the same female were used in experiment and control.*

Although the sperm concentration in certain species combinations to some extent determines the percentage of cross fertilizable eggs (2), this writer is convinced that the said figures have at least an orienting value. Based upon this information two series of experiments were carried out to see whether trypsin would make *Lytechinus* eggs cross fertilizable by spermatozoa from *Mellita sexiesperforata* and *Echinometra lucunter.* In each experiment the control and the experimental sample were inseminated with equal amounts of sperm suspension taken from the same stock suspension in order to ensure that the same concentration of spermatozoa would be present in both. The results are summarized in Tables I–II.

The two series of experiments clearly indicate that the trypsin treatment makes the eggs cross fertilizable and this change in property of the eggs is presumably due to the proteolytic activity of the enzyme molecule.

It appeared that in the presence of trypsin ($^1/_2$ hour in 0.05 per cent) only *0–5.8 per cent* (4 experiments) of *Lytechinus* eggs were fertilizable by sperm from their own species. However, *53.6-93.5 per cent* of the eggs became

TABLE I

Cross fertilization experiments with *Lytechinus* eggs and *Mellita* sperm.

The eggs were kept for $^{1}/_{2}$ hour in 0.05 per cent trypsin, thereafter washed in normal sea water and inseminated		Controls kept and inseminated in normal sea water	
Total number of eggs	Percentage of cleaving eggs	Total number of eggs	Percentage of cleaving eggs
250	26.4	263	1.1
253	55.7	258	1.2
289	34.9	257	0.8
239	28.9	206	1.4
253	68.4	244	1.2

TABLE II

Cross fertilization experiments with *Lytechinus* eggs and *Echinometra* sperm.

The eggs were kept for $^{1}/_{2}$ hour in 0.05 per cent trypsin, thereafter washed in normal sea water and inseminated		Controls kept and inseminated in normal sea water	
Total number of eggs	Percentage of cleaving eggs	Total number of eggs	Percentage of cleaving eggs
269	25.7	227	2.6
260	30.3	212	7.1
271	53.1	210	1.0
222	21.2	205	5.4
179	62.5	248	5.2
246	37.0	203	0.5
264	52.3	239	2.5

fertilizable again after washing in normal sea water. These observations are in agreement with previous findings regarding other sea urchin species (1).

It was of interest to notice that the specimens of *Lytechinus* taken from Harrington Sound were frequently found to contain high percentages of fertilizable eggs up till the end of September thus considerably extending the length of the breeding season given by Harvey (5).

DISCUSSION

As pointed out above Hultin (7, 8) found that a treatment of eggs from several species of Neapolitan sea urchins with an impure trypsin preparation made them cross fertilizable and we may now assume that this is due to the proteolytic activity of the trypsin molecule.

He drew the conclusion that the "barrier" against cross fertilization is located in the vitelline membrane and based this assumption on the observation that the eggs became cross fertilizable subsequent to the removal of the said membrane by the activity of the enzyme. He found, however, that *Arbacia* sperm is unable to fertilize eggs of several species of sea urchins even after removal of their vitelline membranes (8, see also 3). It is furthermore of interest to point out that Hultin's procedure does not exclude the possibility that the trypsin treatment had also changed the structure of the cell surface (the plasma membrane) itself and thereby made the eggs cross fertilizable. On the basis of the evidence reported here we may not go further than to assume that a substance present in or near the cell surface is responsible for the inhibition of the fertilization with sperm cells from the other species and that this substance is inactivated or removed by means of trypsin.

SUMMARY

1. Eggs of *Lytechinus variegatus* are not readily cross fertilizable by sperm from *Mellita sexiesperforata* or *Echinometra lucunter.*

2. However, a treatment of the eggs with crystalline trypsin made them cross fertilizable by sperm from the said species.

3. Fertilization of *Lytechinus* eggs by spermatozoa of their own species is reversibly blocked by trypsin in agreement with previous findings regarding other sea urchin species.

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RESPIRATION, GLYCOLYSIS AND ENERGY RICH PHOSPHORUS COMPOUNDS IN SECRETING AND NON SECRETING RAT SUBMAXILLARY GLANDS

J. F. FERNANDES and L. C. U. JUNQUEIRA

Laboratory for Cell Physiology, Faculdade de Medicina, Universidade de São Paulo, Brasil

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DATA on metabolic phenomena related to cell secretion are scarce in the literature. Attempts have been made to obtain information comparing normal and stimulated glands mainly on pancreas and salivary gland after the action of drugs or electric stimulus (9, 13, 18). Although interesting findings have been reported on this material, it would be convenient to remind here, that a non-stimulated gland cannot properly be considered as a resting gland, due to the presence of secretory processes.

The ligature of excretory duct of mice submaxillary glands induces a decrease in the size of these organs and their cells, accompanied by cytological, histochemical and biochemical phenomena that led us to consider it as a resting gland, but capable of secreting when adequately stimulated (9). Unpublished results from this laboratory indicate that the same behavior is observable in rat submaxillary glands. This is an adequate material to study biochemical processes related to cell secretion when one compares ligated with normal non ligated glands.

In this paper we present the results obtained by comparing the oxygen consumption, anaerobic glycolysis, ATP plus ADP and creatine phosphate, labile phosphorus of normal secreting and ligated glands.

MATERIAL AND METHODS

Adult male rats were used throughout due to the known action of sexual hormones in the rat submaxillary glands (2). Left glands were ligated as previously described (3), remaining the right glands as controls. Experiments were performed in animals ligated 15 to 22 days previously.

Oxygen consumption and anaerobic glycolysis were determined manometrically by the usual Warburg techniques (17). Ringer Krebs phosphate with oxygen atmosphere was used in the oxygen consumption determinations, and Ringer Krebs bicarbonate media plus 200 mg per cent of glucose in $N_2 : CO_2$ 95—5 per cent atmosphere for the glycolysis experiments.

TABLE I

Oxygen consumption and anaerobic glycolysis of control and excretory duct ligated submaxillary gland.

Results expressed as $Q_{O_2}^{O_2}$ DNAP (μl O_2 uptake per μg of desoxyribonucleic acid phosphorus, per hour in oxygen atmosphere) and as $Q_G^{N_2}$ DNAP (μl of CO_2 production per μg of desoxyribonucleic acid phosphorus, per hour, in N_2–CO_2 atmosphere).

Control				Ligated		
Exp.	No.	Number of flasks	Mean	Number of flasks	Mean	Per cent of control
$Q_{O_2}^{O_2}$ DNAP	1	5	5.8 ± 0.4	4	1.5 ± 0.1	26
	2	1	5.7	2	1.8	35
	3	2	4.3	2	2.0	47
	4	1	4.0	1	2.0	50
	5	2	5.0	1	1.8	36
$Q_G^{N_2}$ DNAP	6	6	3.3 ± 0.2	5	3.6 ± 0.1	109
	7	6	3.2 ± 0.1	6	3.1 ± 0.1	97

The animals were killed by a blow on the head, the glands dissected immediately immersed in cold 0.9 per cent NaCl and cut free hand by Deutsch's method (3). All manometric measures were performed at 37° after 10 minutes of preliminary shaking in the water bath.

To measure the ATP plus ADP and creatine phosphate labile phosphorus content, the glands were dissected from nembutal anaesthesized animals and immediately frozen in liquid air. Homogenates of the powdered glands were made in 10 per cent iced TCA solution. Fractionation was performed as described by LePage (10) and ATP plus ADP phosphorus were determined in the barium insoluble fraction after 7 minutes hydrolysis at 100 C in N HCl. Phosphorus was estimated according to Gomori (6).

All results are expressed as related to desoxyribosenucleic acid phosphorus (DNAP), because this procedure probably eliminates errors due to variations in the cellular population of samples, observable in organs that present cell size changes in different conditions (1, 11, 15).

The DNAP was determined in the content of all vessels after manometry, following a modification of Schmidt and Thannhauser's method (16) as described by Rabinovitch (14).

RESULTS

The results obtained studying the oxygen consumption and anaerobic glycolysis are summarized in Table I and Figs. 1 and 2.

A significant decrease of the oxygen consumption and no significant difference in the anaerobic glycolysis are observed in the ligated glands as compared to their controls.

TABLE II

Influence of succinate (0.01 M) upon oxygen consumption of control and excretory duct ligated submaxillary glands.

Result expressed as $Q_{O_2}^{O_2}$ DNAP, meaning μl of O_2 uptake, per μg of desoxyribonucleic acid phosphorus, per hour.

				Succinate		
	Exp. No.	Number of flasks	$Q_{O_2}^{O_2}$ DNAP (mean)	Number of flasks	$Q_{O_2}^{O_2}$ DNAP (mean)	Per cent increase
Control	8	1	5.2	3	8.3	60
	9	2	4.3	3	6.6	53
	10	1	4.0	3	7.9	98
	11	2	5.0	3	7.2	44
Ligated	8	2	1.8	2	2.2	22
	9	2	2.0	2	2.6	30
	10	1	2.0	2	2.5	25
	11	1	1.8	2	2.5	39
	12	1	1.9	4	2.6	37

TABLE III

Influence of succinate and succinate plus malonate upon oxygen consumption of control and excretory duct ligated submaxillary gland.

For method of expressing results see Table II.

	Succinate			Succinate + Malonate		
	Exp. No.	Number of flasks	$Q_{O_2}^{O_2}$ DNAP	Number of flasks	$Q_{O_2}^{O_2}$ DNAP	Per cent decrease
Control	13	3	8.2	3	5.0	39
	14	3	6.4	3	5.8	9
	15	3	5.4	3	3.7	32
Ligated	13	3	2.6	3	1.5	42
	14	3	2.8	3	2.1	25
	15	3	2.4	3	1.5	37

In Table II and Fig. 1 we present the results of the action of succinate (0.01 M) on the oxygen consumption. It can be concluded that the addition of succinate significantly increases the oxygen consumption of the control glands, to a greater extent than the increase observed in the ligated glands.

Influence of malonate (0.03 M) addition on oxygen consumption in presence of succinate can be observed in Table III. One can see that malonate inhibits

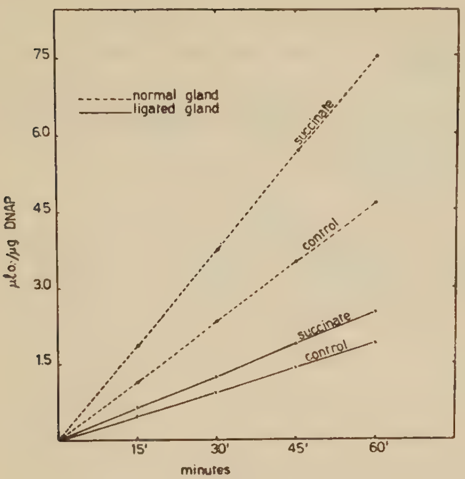

Fig. 1.

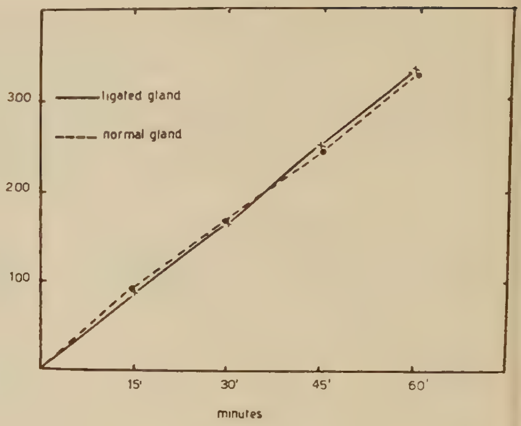

Fig. 2.

Fig. 1. Oxygen consumption in presence of succinate (0.01 M) of control and duct ligated rat submaxillary gland. Results expressed as μl of HO_2/μg of desoxyribonucleic acid phosphorus in oxygen atmosphere.

Fig. 2. Anaerobic glycolisis in slices of normal and secretory duct ligated submaxillary glands. Results expressed as μl of CO_2 production per μg of desoxyribonucleic acid phosphorus in N_2-CO_2 95–5 per cent atmosphere.

Table IV

Inorganic phosphorus, ATP + ADP labile phosphorus (7 min. hydrolysis at 100 min. in N HCl) and phosphocreatine phosphorus content of acid soluble fraction of control and duct ligated submaxillary glands.

Results expressed as μg of phosphorus per 100 μg of desoxyribonucleic acid — phosphorus.

	PI phosphorus	ATP + ADP phosphorus	Phosphocreatine phosphorus
Control	44.8	13.7	8.5
Ligated......................	18.6	3.2	2.7
(per cent of control)	(41.5)	(23.4)	(31.8)

significantly and approximately to the same extent the oxygen consumption of control and ligated glands.

Results obtained from the ATP plus ADP and creatine phosphate labile phosphorus determinations are summarized in Table IV. A distinct and significant decrease of these energy rich phosphorus compounds can be observed.

DISCUSSION

A revision of the literature on submaxillary gland metabolism has been published (9). We would like to discuss here the following aspects pertaining to our results.

The marked decrease of the oxygen consumption of the ligated glands, suggests that the more intense metabolism presented by the control glands can be considered as related to cell secretion. It is interesting to observe that the relatively low oxygen consumption of the ligated glands presents less variation than the controls, probably indicating that these metabolic phenomena in such glands are related to the maintenance of cell structure and life.

Both glands under the action of succinate react by an increase in the oxygen consumption, the control gland however more intensely. This fact suggests a stronger activity of succinic dehydrogenase in the control glands, probably related to phenomena of cell secretion. These results confirm those already published obtained with the Thunberg technique (9). The inhibition induced by malonate gives further evidence that we are dealing with succinic dehydrogenase.

It is of interest to correlate these results with those of Hogeboom *et al.* (7) who localized in the mitochondria the main site of succinic dehydrogenase activity since a marked diminution of mitochondria was observed in the ligated glands (9).

The analysis of the data that were summarized in Table II showed that the slightly larger dispersion of the results obtained in flasks with control glands plus succinate might be due to different amounts of tissues added to a constant concentration of succinate.

It is interesting to note that no significant difference could be observed between the anaerobic glycolysis of control and ligated glands, indicating that this fermentative process is unaffected by cell secretion. One might remember here that the glycolytic activity has been localized mainly in the soluble fraction of the cytoplasm (8) and showed in our material no variation in spite of the marked cytoplasm decrease per cell.

The maintenance of the glycolytic activity of the ligated glands, in spite of the decrease of its cytoplasm content, might be due to an unequal ATP-ase activity in control and ligated glands (12). The results of DuBois and Potter (5) who found an intense ATP-ase activity in the rat submaxillary glands strengthens this reasoning; however a preliminary ATP-ase assay by DuBois and Potter's method (5), showed no marked difference in the ATP-ase activity between control and ligated glands.

The decrease of the oxygen consumption, and the maintenance of the anaerobic glycolysis of the ligated glands suggests that this metabolic pathway probably provides these glands with a relatively larger proportion of energy than the glycolysis of control glands.

The significant decrease of ATP plus ADP and creatine phosphate labile phosphorus suggests that the energy from energy rich phosphorus compounds might be related to processes of cell secretion.

SUMMARY

Oxygen consumption, anaerobic glycolysis, ATP plus ADP and creatine phosphate labile phosphorus were studied in normal (secreting) and duct ligated (resting) rat submaxillary glands.

Oxygen consumption, phosphocreatine and ATP plus ADP labile phosphorus were significantly decreased in the ligated glands as compared to normal controls. No significant change in the anaerobic glycolysis could be observed between these glands. The significance of these results is discussed.

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ELECTRON MICROSCOPE STUDIES OF SILVER SALMON SPERMATOZOA (*ONCORHYNCHUS KISUTCH* [WALBAUM])[1]

F. G. LOWMAN

Applied Fisheries Laboratory, University of Washington, Seattle, Wash., U.S.A.

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During a series of studies concerning the effects of X-rays upon the gametes and embryological stages of the rainbow trout, *Salmo gairdnerii* (Walbaum), the need for more detailed knowledge of the morphology of salmonoid spermatozoa became apparent; consequently an electron microscope study was undertaken. At that time spermatozoa of the silver salmon, *Oncorhynchus kisutch* (Walbaum), were available and were used for the electron microscope study described in this paper.

Because of the possibility of producing artefacts during preparation of the material, several different fixatives with and without buffer were used. The appearance of the spermatozoa during each step of fixation and preparation of electron microscope grids was checked by means of the light microscope, with the appearance of fresh unfixed spermatozoa serving as the control. It was hoped that this method might be used for detecting the occurrence of artefacts in variously fixed material, which was found to be the case in some instances. Often, however, differences in the structure of the spermatozoa were seen with the electron microscope although no change had been evident with the light microscope. It was apparent that the use of the latter for determining the presence of artefacts was limited and that the true structure might be more accurately ascertained by comparative electron microscope examinations of spermatozoa which had been fixed with different fixatives and techniques.

MATERIALS AND METHODS

The spermatozoa were obtained by stripping mature silver salmon which had been released as fingerlings in the spring of 1950 from the Fisheries Center, University of Washington, and which had returned from the sea in the fall of 1951. Care was taken to prevent water from being introduced into the milt, and fixation was usually accomplished within a few minutes after the milt was procured.

The spermatozoa were fixed by placing a drop of fresh, undiluted sperm into a Syracuse watch glass half-full of the desired fixative and mixing rapidly by light shaking. The following buffer-fixative combinations were used:

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[1] This report is based upon work performed under Contract Number AT(45–1)540 with the Atomic Energy Commission.

1. Water; 2. Equal parts of Michaelis' (11) buffer[1] and 1 per cent osmium tetroxide; 3. Neutral formalin (10 per cent); 4. Equal parts of Michaelis' buffer and neutral formalin (10 per cent); 5. Michaelis' buffer and acetic acid vapor; 6. Equal parts of Michaelis' buffer and 5 per cent acetic acid; 7. Equal parts of Michaelis' buffer and alcohol (50 per cent); 8. Saturated picric acid; 9. Equal parts of Michaelis' buffer and saturated picric acid; 10. Saturated mercury bichloride; 11. Equal parts of Michaelis' buffer and saturated mercury bichloride; 12. Equal parts of Michaelis' buffer and Zenker's with 5 per cent acetic acid; 13. Equal parts of Michaelis' buffer and Zenker's (no acetic acid); 14. Zenker's with 5 per cent acetic acid; 15. Zenker's (no acetic acid); 16. One part Michaelis' buffer, one part 1 per cent osmium tetroxide and two parts Zenker's with 5 per cent acetic acid; 17. Equal parts of 1 per cent osmium tetroxide and Zenker's with acetic acid; 18. One part 1 per cent osmium tetroxide, one part Michaelis' buffer and two parts Zenker's with 5 per cent acetic acid; 19. Equal parts of 10 per cent neutral formalin and Zenker's with 5 per cent acetic acid; 20. One part Michaelis' buffer, one part 10 per cent neutral formalin, and two parts Zenker's with 5 per cent acetic acid.

In addition to these following enzymes were used:

21. Ribonuclease ($^1/_{10}$ mg/cc aqueous dilution digested at 25° C). Durations of digestion: a. No digestion (control), b. 10 minutes, c. 20 minutes, d. 50 minutes, e. 60 minutes, f. 90 minutes.

22. Desoxyribonuclease. Durations of digestion: a. 60 minutes, b. 90 minutes.

Since fertilization occurs in water in the case of salmonoids, some of the samples were mixed with water before the fixative was added. Most of the spermatozoa thus fixed exhibited the same structural characteristics as those fixed without previous addition of water. However, because of the difficulty in allowing exactly the same time interval each time between the addition of water and the start of fixation, the method was not used extensively.

The electron microscope screens of 200-mesh nickel were prepared as follows. New 1″ × 3″ glass microscope slides were washed in 95 per cent ethanol and wiped clean with lens tissue. A drop of 1 per cent Formvar E[2] in ethylene dichloride was placed on one slide and another slide immediately placed on top with a two-inch overlap of the slides. The slides were then rubbed together with a one-eighth-inch rotary motion and, after the formvar solution was spread evenly, the two slides were quickly slid apart in the direction of their long axes. The film remaining on each slide dried very rapidly. By breathing lightly upon the film and immediately but slowly immersing the slide at an acute angle into lint-free water, it was possible to float the formvar film off the slide onto the water surface. Light interference fringes could now be seen in the film and were utilized to select areas of less than 200 Å thickness. The slide was kept under the water surface and the nickel screens placed on it. The slide was then used to lift the film back out of the water, with the desired area of the film having been moved into position over the screens, and placed under a petri dish lid on a blotter to dry.

After fixation a drop of the suspension of spermatozoa was placed upon the formvar film supported by the nickel screen. The spermatozoa settled and became attached to the film in 10 to 15 minutes. Excess fixative and seminal fluid were then removed by alternate washes with 1 per cent NaCl solution and tap water applied with an eyedropper. Preparations in which distilled water was used instead of tap water[1] were not found to be superior in any way, therefore, distilled water was not used. Duration of washing was determined by examination of the screens under a compound microscope at a magnification of 100. When 2 to 10 spermatozoa could be seen in each mesh of the screen, washing was stopped. After the washing period, with water used as the final wash, the grids were placed under petri dish lids on blotters and allowed to dry. This method of preparation resulted in unusually clean samples with almost no foreign matter clinging to the spermatozoa.

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[1] Only Michaelis' acetate-veronal buffer at a pH of 7.4 was used in this work.

[2] Available from Shawinigan Products Corporation, New York, New York.

After the film with fixed spermatozoa had dried at least 24 hours the preparations were shadowcast. During the first part of the study chromium shadowed at an angle of 18.5° (3:1) was used; later chromium and palladium were used at an angle of 45° (1:1). Chromium was found to be adequate at low and intermediate magnifications (Plate I, Fig. 1, Plate IV, Figs. 1 and 3, and Plate V, Fig. 2), but was not satisfactory at high magnifications. Palladium, with an atomic weight approximately twice that of chromium, appears essentially structureless even at the highest magnifications (16), and layers of palladium 5 Å to 10 Å thick resulted in sufficient contrast to bring out fine detail.

The microscope used was an RCA model EMU-2A leased from the University of Washington Engineering Experiment Station. It was operated at an accelerating potential of fifty KV and a beam current of .1 MA to .3 MA. Dow latex 580-G was photographed in the microscope to determine the magnification at the different steps. Kodak standard lantern slide plates (medium and contrast) were used and were developed 4 minutes at 20° C in undiluted stock Kodak D-19. Intermediate negatives were made by projection on Kodalith Orthochromatic Type 2 film or on Kodak standard lantern slide (medium and contrast) plates. These were developed in Kodak Dektol developer diluted with two parts of water. Prints were made from the intermediate negatives on Kodabromide paper.

General Structure

The spermatozoon of the silver salmon may be divided into three regions: the head, neckpiece, and tail (Plate IV, Fig. 1, Plate V, Fig. 2).

The head. The head of the spermatozoon in normal condition is egg-shaped with the acute end directed anteriorly. It has a long axis of approximately 2.0 μ and a maximum width of approximately 1.5 μ. The outline of the head is not that of a true ovoid but is almost always more or less asymmetrical posteriorly (Plate I, Figs. 1 through 7).

Because of its marked opacity to the electron beam no detail within the head could be detected, nor was there any evidence on the surface of an acrosome or post nuclear cap.

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[1] Total hardness of the tap water was 19.8 parts per million.

The neckpiece. The neckpiece is joined to the tail near the origin of the latter at the head. The main body of the neckpiece is ovoid in shape with its long axis lying perpendicular to the axis of the tail. It is located to one side of the tail. Its dimensions are approximately 0.4 μ to 0.7 μ although its size and shape vary with the use of the different fixatives. When fixatives that result in marked hardening of protein are used (i.e., saturated aqueous picric acid, saturated aqueous mercury bichloride, or Zenker's fixative) the neckpiece appears small and globular (Plate I, Figs. 2 and 5). With other fixatives the neckpiece becomes flattened against the supporting film and more variable in shape (Plate I, Figs. 1, 6, and 7, Plate II, Figs. 4 and 6). In Plate V, Fig. 2, the neckpiece has been pulled away from the tail and the latter has been split with part of it remaining attached to the neckpiece. In Plate I, Fig. 3, the neckpiece has been pulled away from the head. Here there is no indication of any connection between the head and neckpiece but there is continuity of the neckpiece and tail. Plate I, Fig. 5, further substantiates the lack of continuity between the neckpiece and the head.

The tail. The tail consists of an axial filament covered by a spirally wound, membranous sheath of less than 100 Å thickness (Plate II, Figs. 1, 2, 3, Plate III, Figs. 4, 5, 6, and 7). The axial filament is composed of eleven fibrils of which nine are greater in diameter than the remaining two. The nine larger fibrils are arranged lengthwise to form a cylinder in the center of which lie the two smaller fibrils (Plate IV, Fig. 2). All of the fibrils lie parallel to each other and to the longitudinal axis of the axial filament.

The axial filament is of constant diameter (approximately 1600 Å) from its origin at the head to a point approximately 2 μ to 3 μ from the tip of the tail. Here it is reduced to a diameter of approximately 1000 Å which is constant over a length of approximately 1 μ, where it is again reduced to a diameter of 300 Å to 600 Å. Its diameter is constant for the remaining 1 μ to 2 μ of the tip of the tail (Plate IV, Fig. 1).

Fine Structure of the Tail

The sheath. The sheath makes eleven to fifteen spiral turns around the axial filament from the neckpiece to the point where the tail first becomes reduced in thickness (Plate II, Figs. 1, 2, and 3). Unlike the sheath of the spermatozoan tail of the domestic fowl (6) the tail sheath is not thicker at the anterior end but is of uniform thickness throughout its length, with the exception of a very thin portion surrounding the terminal 1.5 μ to 3.0 μ of the tail. This portion is so thin that with ordinary fixing techniques it is usually destroyed. That the thin terminal sheath is a continuation of the thicker sheath is indicated in Plate III, Fig. 2.

The axial filament. The following fixatives tend to cause fraying of the fibrils in the tip of the tail (Plate II, Fig. 5, Plate III, Fig. 3, Plates VI, VII, and VIII):

1. Equal parts of Michaelis' buffer and 1 per cent aqueous osmium tetroxide; 2. Ten per cent neutral formalin; 3. Equal parts of Michaelis' buffer and 10 per cent neutral formalin; 4. One part Michaelis' buffer, one part 1 per cent aqueous osmium tetroxide and two parts Zenker's fixative with acetic acid; 5. Equal parts of Michaelis' buffer and saturated aqueous mercury bichloride; 6. Equal parts of Michaelis' buffer and saturated aqueous picric acid; 7. Michaelis' buffer and acetic acid vapor.

When any of the first four of the above combinations are used disruption of the tail can occur at any place throughout its length, with the sheath bursting and the fibrils becoming separated (Plate III, Fig. 4, Plate IV, Fig. 3, Plate V, Figs. 1, 2, 3, 4, and 5). Rötheli and Roth (14) refer to the disruptions that occur at the bends in the tails as "kinoplasmic bubbles" and state that they are important structures in the movement of the spermatozoon, since they found them only in spermatozoa which had been fixed during their motile stage. In the present work these structures were found in spermatozoa which were fixed before as well as during their motile stage, and it is evident in Plate V, Figs. 1, 3, and 4 that the disruptions at the sharp bends are fixation artefacts involving the tail fibrils. That the structures described by Rötheli and Roth were artefacts is suggested by the fact that they found the "kinoplasmic bubbles" after the use of Zenkers' and Apathy's fixatives but not in material which had been fixed only by drying.

Each fibril of the silver salmon spermatozoon is isodiametric throughout its length. This is in agreement with the findings in ram (13), fowl (6), and *Arbacia* (7) spermatozoa, though not with those in bull, horse, and dog spermatozoa, in which the fibrils are of greater diameter near their origin and gradually decrease in diameter to the end of the tail (2).

In the disrupted portion of an axial filament shown in Plate III, Fig. 1, ten fibrils can be counted. One of the fibrils (M), which may be composed of two smaller fibrils, is of greater diameter than the others. Other evidence that not all of the fibrils are equal in diameter is found in the tips of the tails that have been frayed during fixation. The tips of several spermatozoan tails are shown in Plate VI, Figs. 2, 3, and 4 b, Plate VII, Figs. 1 and 2 a, and Plate VIII. Four of the fibrils are longer than the others, thus forming the reduced tip of the tail. Two of these fibrils extend approximately 1.0 μ beyond the point of termination for the majority of the fibrils, and the other two longer fibrils extend farther by 1.5 μ to 2.0 μ to form the narrowest part of the tail. These two longest fibrils have a diameter of approximately 300 Å and the others a diameter of approximately 400 Å. If the wider fibril in Plate III, Fig. 1, is composed of the two longest fibrils then there are eleven fibrils in the axial filament.

Grigg and Hodge (6) classified the fibrils of the axial filament into two categories, the L fibrils and the M fibrils. The L fibrils were designated as those which are uniform and equal in diameter, whereas the remaining two, the M fibrils, were found to be more susceptible to destruction by distilled water and to be variable in diameter. If their published photographs are representative of the M fibrils, it is also true that the M fibrils are of smaller diameter than are the L fibrils. These authors do not indicate whether the M fibrils are longer than the L fibrils, thus forming the fine tip of the tail.

Although the M fibrils of the spermatozoa of the domestic fowl may not be homologous with the two longest, thinnest fibrils of the salmonoid spermatozoon, the classification of the fibrils into the categories of Grigg and Hodge will be used in this paper with one minor exception. The two thinnest fibrils will be designated M fibrils, the other two fibrils of intermediate length, the L_1 fibrils and the remaining seven, the L fibrils.

Schmitt (15) in a discussion of contractile fibers described the tail fibril as the unit of structure in the organization of the spermatozoan tail but stated that "there is no doubt that it is composed of still finer columnar units; occasionally finer strands are seen." Bretschneider and Van Iterson (3) in a study of bull spermatozoa detected a splitting of the fibrils into protofibrils but did not report the diameter of the protofibrils. Grigg and Hodge (6) found in trypsin-treated specimens of spermatozoa from the common fowl many fine fibrils 50 Å to 100 Å in diameter. Hodge (8) detected a periodic cross striation in the tail fibrils of the human spermatozoon with an axial period of approximately 100 Å. Randall and Friedlaender (13) in an investigation of ram spermatozoa found the axial filament to consist of twelve fibrils, each fibril consisting of two filaments (protofibrils) surrounded by a two-ply helix.

Since it has been shown by Grigg and Hodge (6) with fowl spermatozoa and by the present author with salmon spermatozoa that the L and M fibrils are different at least in size, the fibril hardly can be considered the unit of structure of the spermatozoan tail. In view of the differential susceptibility of the L and M fibrils to destruction by distilled water as well as by pepsin (2) it may well be that even the protofibrils are not the unit of structure of the spermatozoan tail.

Although Grigg and Hodge (6) did not observe any regular small structure within the tail fibrils of the common fowl, they did find an irregular and ill-defined periodicity along the length of the fibrils lying in the range of 150 Å to 200 Å. In other samples they found a periodicity in the fibrils with a period of approximately 1000 Å. In the present work with silver salmon several samples which had been fixed in 10 per cent formalin plus buffer showed ill-defined bands along the fibrils where the sheath had been disrupted (Plate III, Figs. 4 and 7, and Plate IV, Fig. 3). The above findings suggested either that each fibril was a single unit 300 Å to 400 Å in diameter with periodic swellings along its length or that each fibril consisted of two or more protofibrils irregularly coiled around each other. Because of the variable periodicity and the fact that other authors had demonstrated the presence of occasional protofibrillar elements, it appeared more probable that the fibrils were composed of two or more protofibrils. Subsequent detailed studies supported this view.

Fibrillar detail shows well following the fixation of fresh, undiluted milt of silver salmon with equal parts of Michaelis' buffer and 1 per cent aqueous osmium tetroxide if this mixture is then combined with an equal amount of Zenker's fixative (with 5 per cent acetic acid). In several samples of sperm fixed in this manner it was noted that some of the spermatozoa developed vesicles at the tips of the tails (Plate VI, Fig. 4 b). Closer examination showed that the vesicles were composed, in part at least, of the ends of fibrils which had been disrupted during fixation. In several instances the unraveling of the fibrillar substructures, the protofibrils, with consequent formation of vesicles could be seen (Plate VI, Figs. 4 a and 4 b, Plate VII, Fig. 2 a (P), Plate VIII (P)). Unraveling of the protofibrils is also shown in Plate VI, Fig. 2 (P), and Plate VII, Fig. 1 (P), and Fig. 2 b (P), although it is not as extensive as in the previous examples.

Evidence of the presence of protofibrils is found within the intact portions of the fibrils as well as at their frayed ends. In Plate VI, Fig. 1, a clockwise spiral substructure within the M fibrils can be seen. In the enlarged inset of the same figure, it appears that the fibril is composed of two protofibrils. Within other M fibrils (Plate VI, Fig. 3, Plate VII, Fig. 1, and Plate VIII) there are indications that from two to four protofibrils comprise the fibrils, although the photographs are not sufficiently clear for accurate determination of the number. The normal number of protofibrils in the M fibrils, however, is probably four and in fibrils showing less than this number the protofibrils which are missing have broken and unraveled. In all fibrils examined the protofibrils exhibited a clockwise spiraling and could be seen only when palladium was shadowed from a source approximately perpendicular to the spirals.

In Plate VI, Fig. 3, the inset shows an enlarged section of the lower M fibril. Here two of the protofibrils have coalesced, probably as a result of the fixation process, resulting in the configuration indicated in Textfig. 1a. If the protofibrils were to pair and coalesce over any appreciable length of the fibrils, the configuration shown in Textfig. 1b would result with an apparent period of about 500 Å. If all four fibrils coalesced, a period of about 1000 Å would result (Textfig. 1c). A period of 1000 Å was found by Grigg and Hodge (6) in single fibrils and in apparently intact tails of spermatozoa of the common fowl, although they did not find the cross striation to be a reproducible phenomenon. If the configuration shown in Textfig. 1c is the cause of the 1000 Å periodicity, then one would not expect that it would be produced consistently since it is probably an artefact due to technique. Harvey and Anderson (7) working with *Arbacia* spermatozoa found regularly spaced cross striations along the tail in certain areas. They do not state the interval of the period but from their photograph of the structure it appears that the interval is between 500 Å and 800 Å. Kraneveld, Houwink, and Keidel (10), in an electron microscope investigation of trypanosomes, found the flagellum to consist of approximately nine isodiametric fibrils 400 Å to 500 Å in diameter. They also detected fine cross striations with a period of 500 Å along the flagellum but rejected the idea that they were due to a periodicity in the structure of the fibrils since the fibrils looked smooth when isolated. Further studies should indicate whether the cross striations of 500 Å and 1000 Å periods in fibrils of spermatozoa and protozoa are due to the configurations shown in Textfigs. 1b and 1c or to some other structure.

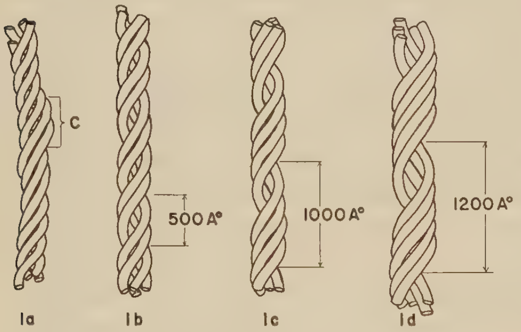

Fig. 1. The probable configuration of the M fibril shown in Plate VI, Fig. 3, is indicated. Two of the protofibrils have coalesced (c). b. The four protofibrils of an M fibril have coalesced into two groups of two; consequently, a 500 Å period is seen along the length of the fibril. c. All of the protofibrils of an M fibril have coalesced into a single group with a resulting period of approximately 1000 Å occurring along the length of the fibril. d. The five protofibrils of an L or L_1 fibril have coalesced into a single group. A period of approximately 1200 Å along the length of the fibril is indicated.

The maximum number of protofibrils found in the L_1 fibrils is five. This was determined by measurement of the pitch and approximate width of the coils within the L_1 fibrils in photographs such as Plate VII, Fig. 2, and Plate VIII.

The number of protofibrils comprising each of the seven L fibrils has not been determined. The presence of substructure within the fibrils cannot be determined by shadowcasting in that portion of the tail covered by the 100 Å sheath, since the inter-protofibrillar spaces are covered by the sheath.

Effects of Fixatives on the Head

The shape and size of the spermatozoan head are not constant and are affected by the type of fixative used, the osmotic pressure of the fixing fluid at the time of fixation, and the surface tension of the water exerted upon the spermatozoa during drying of the preparation. The third factor is constant since water was always used for the final wash.

An insufficient number of measurements was made for a quantitative study of the effects of osmotic stress and chemical hardening upon the head. However, a qualitative evaluation can be made.

In Textfig. 2, the average dimensions of the head are plotted for each of the fixatives and fixative-buffer combinations used.

The degree to which the spermatozoan head is changed in size under osmotic stress is limited by the hardening ability of the fixative and by the rate at which hardening occurs. Of the fixatives used picric acid exerts the greatest hardening action upon the spermatozoan head, with the following fixatives exerting their effect in decreasing order:

1. Zenker's fixative; 2. Formalin; 3. Alcohol; 4. Osmium tetroxide.

Based upon the degree of flattening of the tail, i.e., the less flattening the

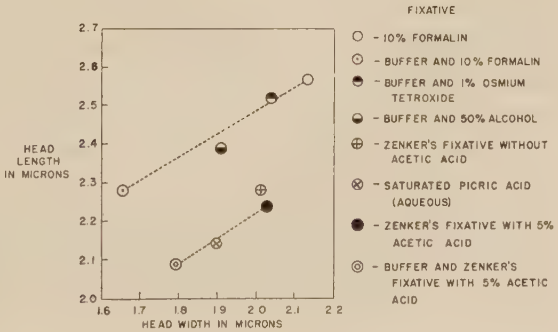

Fig. 2. Averaged spermatozoan head lengths are plotted against averaged head widths to indicate the effects upon head dimensions of different fixative and fixative-buffer combinations.

TABLE I

Fixative	No. of Measurements	Average Tail Width	Individual Tail Length
10 per cent neutral formalin	11	(217 ± 12.7) × 10 Å	—
Buffer and 10 per cent formalin	6	(211 ± 15.7) × 10 Å	—
Buffer and 1 per cent osmium tetroxide	28	(267 ± 7.3) × 10 Å	31.4 μ; 28.0 μ
Saturated picric acid	4	(112 ± 5.2) × 10 Å	—
Buffer and saturated picric acid	4	(167 ± 6.5) × 10 Å	32.3 μ
Buffer and acetic vapor	6	(168 ± 7.6) × 10 Å	—
Buffer and 50 per cent alcohol	6	(164 ± 6.3) × 10 Å	—
Zenker's (no acetic acid)	3	(147 ± 7.6) × 10 Å	32.0 μ
Buffer and Zenker's (no acetic acid)	2	(156 ± 5.0) × 10 Å	—
Zenker's with acetic acid	4	(143 ± 5.5) × 10 Å	28.9 μ
Buffer and Zenker's with acetic acid	9	(134 ± 10.5) × 10 Å	34.6 μ
Osmium tetroxide and buffer and Zenker's with acetic acid	9	(183 ± 15.7) × 10 Å	—

more hardening, the fixatives harden in the same relative order except that formalin and alcohol are in reverse order.

When spermatozoa were fixed with the same fixative with and without buffer, the effect of osmotic stress upon the observed size of the head was apparent. The increased osmotic pressure of the fixing medium, afforded by the buffer, resulted in less swelling of the spermatozoan heads than when the fixatives were used without buffer.

An attempt was made to reduce the opacity of the head with nucleases. Digestion of the spermatozoa with ribonuclease and desoxyribonuclease for periods up to 90 minutes resulted in no reduction of opacity.

Effect of Fixatives on Tail Structures

The observed dimensions of the tail are dependent upon the degree of hardening caused by the fixative and, to a lesser degree, upon the amount of unraveling of the substructures of the tail during fixation and washing. Both of these factors are affected by the fixatives used and by the presence or absence of buffer at the time of fixation. The tail lengths vary from 28 μ to 35 μ and the tail widths from .110 μ to .265 μ.

The average tail widths resulting from the use of several different fixatives are given in Table I. The tail lengths are indicated in those instances where they were obtained, although the use of different fixatives did not result in significant differences in length.

The variation in tail width with different fixatives does not fall within the expected range of variation, thus significant differences in width do exist. In addition there is a highly significant difference in one instance between the effects of a fixative with and without buffer, namely saturated picric acid. This is the only fixative used in which the addition of the buffer resulted in any great difference in tail width. In Table II a, four fixatives without buffer are compared to determine if any significant differences exist in their effect upon tail width. In Table II b, eight fixatives with buffer are compared for the same determination.[1] Student's T test was used to determine the significance of the differences.

Because significant buffer effect regarding tail width was found only with picric acid, it is apparent that any minor variations in tail width with the use or nonuse of buffer with the other fixatives are due to structural or physiological variations in individual spermatozoa at time of fixation. Thus it would be expected that tail widths would not differ significantly with any two fixatives except picric acid, when the same two fixatives were used with buffer. With two exceptions this has been found to be true. (Tables II a and II b.) The exceptions involve the comparisons of 1) Zenker's fixative without acetic acid with 10 per cent formalin, and 2) Zenker's fixative without acetic acid with Zenker's fixative with acetic acid, with the same combinations plus buffer. Because only three measurements were taken for Zenker's without acetic acid and two measurements for buffer and Zenker's without acetic acid, these apparent discrepancies are not of great importance.

If the degree of flattening of the tail be taken as an index of hardening action, 1 per cent osmium tetroxide and buffer is the least hardening and saturated picric acid without buffer is the most hardening of the fixatives used. Buffer and Zenker's with acetic acid results in an average tail width which is significantly less than any of the other buffer-fixative combinations used, although it is greater (highly significant) than that resulting from the use of saturated picric acid alone.

With the exception of 1) one per cent osmium tetroxide and buffer, 2) buffer and Zenker's with acetic acid, and 3) saturated picric acid, all fixatives tested resulted in tail widths that fell within the range of expected variation. This does not indicate, however, that equal fixation of the tail structure occurs in all of these cases. That this is not true is evidenced by the difference in detail observed in the tail substructure when these apparently equal-acting fixatives are used.

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[1] Four fixatives were tested with buffer that were not tested without buffer. This was necessary because of the excessive clumping of the spermatozoa when buffer was not used with these fixatives.

Brown (4) in a discussion of fixatives refers to osmium tetroxide as the "best killing and fixing reagent for retaining original appearances" in protozoan flagella. However, in the present work it soon became apparent that the advantageous qualities of osmium tetroxide fixation would be of little value unless the marked flattening of the fixed structures could be overcome (Plate V, Fig. 5). On the other hand, fixatives which were strong precipitants were of limited use because of the coagulation artefacts which they produced. If initial fixation with osmium tetroxide and subsequent fixation by a more drastic fixative could be accomplished, the detail obtainable with osmium tetroxide might be retained and the problem of flattening solved as well. To test this possibility the spermatozoa were fixed first with buffer and osmium tetroxide and then by Zenker's with acetic acid.

The average tail width resulting from fixation with buffer plus osmium tetroxide was 2670 Å. When the spermatozoa were subsequently refixed

TABLE II

AVERAGE TAIL WIDTH	FIXATIVE				
2170 A°	10% FORMALIN	—			
1470 A°	ZENKER'S WITHOUT ACETIC ACID	● P=<2%	—		
1430 A°	ZENKER'S WITH ACETIC ACID	●● P=<1%	○ P >50%	—	
1120 A°	SATURATED PICRIC ACID (AQUEOUS)	●● P=<1%	● P=<2%	●● P=<1%	—
		10% FORMALIN	ZENKER'S WITHOUT ACETIC ACID	ZENKER'S WITH ACETIC ACID	SATURATED PICRIC ACID

IIa. Four fixatives are compared with each other with reference to their effect upon tail width.

○ – NO SIGNIFICANT DIFFERENCE IN TAIL WIDTH

● – SIGNIFICANT DIFFERENCE IN TAIL WIDTH

●● – HIGHLY SIGNIFICANT DIFFERENCE IN TAIL WIDTH

AVERAGE TAIL WIDTH	FIXATIVE							
2670A°	BUFFER AND 1% OSMIUM TETROXIDE	—						
2110 A°	BUFFER AND 10% FORMALIN	●● P=<1%	—					
1830 A°	BUFFER, 1% OSMIUM TETROXIDE AND ZENKER'S WITH ACETIC ACID	●● P=<1%	○ P=<30	—				
1680 A°	BUFFER AND ACETIC ACID VAPOR	●● P=<1%	● P=<5%	○ P=<50%	—			
1640A°	BUFFER AND 50% ALCOHOL	●● P=<1%	● P=2%	○ P=<40%	○ P=>50%	—		
1670 A°	BUFFER AND SATURATED PICRIC ACID	●● P=<1%	○ P=<10%	○ P=>50%	○ P=>50%	○ P=>50%	—	
1560A°	BUFFER AND ZENKER'S WITHOUT ACETIC ACID	●● P=<1%	○ P=<10%	○ P=<50%	○ P=>50%	○ P=>50%	○ P=<40%	—
1340 A°	BUFFER AND ZENKER'S WITH ACETIC ACID	●● P=<1%	●● P=<1%	●● P=<1%	●● P=<1%	●● P=<1%	●● P=<1%	● P=<2%
		BUFFER & 1% OSMIUM TETROXIDE	BUFFER & 10% FORMALIN	BUFFER, 1% OSMIUM TETROXIDE & ZENKER'S WITH ACETIC ACID	BUFFER & ACETIC ACID VAPOR	BUFFER & 50% ALCOHOL	BUFFER & SATURATED PICRIC ACID	BUFFER & ZENKER'S WITHOUT ACETIC ACID

IIb. Eight buffered fixatives are compared with each other with reference to their effect upon tail width.

by Zenker's with acetic acid, the average width was 1830 Å. That there was an initial fixation by the osmium tetroxide which was not entirely altered by subsequent fixation with Zenker's and acetic acid is indicated by the widths of the tails fixed by buffer and Zenker's with acetic acid, with no previous fixation by osmium tetroxide. Here the tail width was 1340 Å. All three widths vary significantly from each other. The osmium tetroxide accomplished initial fixation which was not entirely altered by the subsequent re-fixation. For this reason a detailed study with double fixation was undertaken.

Fixatives used for this work the results of which were not described are:

1. Water; 2. Aqueous saturated mercury bichloride; 3. Equal parts of Michaelis' buffer and saturated mercury bichloride; 4. Equal parts of 1 per cent osmium tetroxide and Zenker's with 5 per cent acetic acid; 5. Equal parts of 10 per cent formalin and Zenker's with 5 per cent acetic acid.

With all of the above materials the main structures of the spermatozoa were evident, although fixatives 2 and 3 caused excessive hardening and distortion of the neckpiece and tail structures. Omission of pictures taken after fixation with techniques 1, 4, and 5 of the above list does not indicate that fixation was poor but rather that pictures with other fixatives happen to show specific structures with greater clarity.

DISCUSSION

Although little has been learned of the substructure of the head and neckpiece thus far by use of the electron microscope, the instrument has been especially useful for determining the morphology of the tail.

There is agreement among different authors that the tail of the spermatozoon is the site of conversion of chemical energy into mechanical energy but beyond this there is marked disagreement. Houwink (9), in answer to a suggestion of Pitelka (12) that the axoneme (axial filament) probably is the contractile organ, wrote "With this conclusion we do not agree . . . Looking at the matter from the viewpoint of mechanics one would expect the axoneme to be an elastic skeleton, but the sheath to contain contractile elements." In those instances where the thick sheath is composed of helically wound fibrils, as in mammalian spermatozoa, the concept of the sheath as the contractile structure is attractive. However, in salmon spermatozoa and to a lesser degree, fowl spermatozoa (6), the thinness of the sheath, its frangibility, and the absence of helically wound fibrils raise serious doubts as to whether such a fragile structure would be capable of propelling the spermatozoon.

Schmitt (15) states that the carbohydrate metabolism of muscle and the spermatozoan tail resemble each other qualitatively but differ in the nature of their fibrous proteins and in their motion, with the muscle exhibiting longitudinal shortening and the spermatozoan tail undulatory motion. Under certain conditions it might be possible for longitudinal shortening to be translated into undulatory motion and without any specialized, central skeletal structure. This possibliity has been discussed briefly by Hodge (8).

If a series of parallel, fibrous, contractile fibrils were laterally associated to form a sheet-like structure and this rolled up to form a tube parallel to the axes of the fibrils, the tube would present an undulatory spiral motion if the stimulus to contract were transmitted to the origin of each fibril consecutively and if the contraction passed down each fibril as a relatively long-period wave. The degree of success of the production of motion would, of course, depend upon the rigidity of each fibril during the non-contracting stage as well as upon its ability to contract. The resulting direction of travel of the tube would be toward the origin of the stimulus and since the contractile wave would travel around the tube as well as along it, the resulting helix would rotate in the opposite direction to that of the contractile wave and to that of the head. Examination of live spermatozoa with the light microscope verifies the fact that the heads rotate on their own axes during forward movement.

The nature of the stimulus for contraction is highly conjectural. However, it has been shown by Cody (5) that in spermatozoa of the guinea pig the stimulus originates just behind and not inside of the head.

SUMMARY

The electron microscope has been used to study the structure of spermatozoa of the silver salmon, *Oncorhynchus kisutch* (Walbaum). Several fixative and fixative-buffer combinations were used to differentiate artefacts from actual structures.

Because of the opacity of the head to electrons little was learned of its internal structure. Changes in head dimensions, however, were found with differences in osmotic stress and degree of hardening at the time of fixation.

The neckpiece is attached to and lies lateral to the tail at its insertion into the head. It is an ovoid structure, the size of which varies with the use of different fixatives.

The tail is composed of an isodiametric axial filament containing eleven fibrils and is covered by a spirally wound, membranous sheath. Nine of

the fibrils are of equal diameter and are probably each made up of five protofibrils. The remaining two fibrils are of smaller diameter than the others and contain four protofibrils each. The protofibrils comprise the clockwise spiral substructure of the fibrils, in which no central core was detected.

An analysis of the effects of different fixatives upon the tail dimensions was attempted. In some instances highly significant differences in tail widths with different fixatives were found.

I am indebted to my colleagues of the Applied Fisheries Laboratory for helpful advice, suggestions, and aid in procuring materials for this work. To Mr. Oliver Row, electron microscopist for the Engineering Research Station, I am especially grateful. Without his cooperation and technical ability this work would not have been possible.

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PLATES

Treatment — the following list includes those treatments referred to in the plate texts.

1. Michaelis' buffer and 1 per cent osmium tetroxide; 2. Ten per cent neutral formalin; 3. Michaelis' buffer and 10 per cent neutral formalin; 4. Michaelis' buffer and 50 per cent alcohol; 5. Michaelis' buffer and saturated picric acid; 6. Michaelis' buffer and Zenker's fixative with acetic acid; 7. Zenker's fixative with acetic acid; 8. Zenker's fixative without acetic acid; 9. Michaelis' buffer, 1 per cent osmium tetroxide and Zenker's fixative with acetic acid; 10. Chrome shadowed at an angle of 18.5°; 11. Chrome shadowed at an angle of 45°; 12. Palladium shadowed at an angle of 45°.

Plate I

Fig. 1. Treatment, 1 and 10.
A silver salmon spermatozoon with a head, neckpiece (n.p.), axial filament (a.f.), and tail sheath (s.) is shown. The sheath has been disrupted and lies on each side of the axial filament.
Fig. 2. Treatment, 8 and 10.
The neckpiece (n.p.) is small and globular. The axial filament is dense and round in cross section. The tail sheath (s.) surrounds the axial filament.
Fig. 3. Treatment, 9 and 12.
The neckpiece (n.p.) has been torn from its normal position. Connections between the neckpiece and the tail are shown.
Fig. 4. Treatment, 6 and 11.
The neckpiece (n.p.) is small and globular and the axial filament (a.f.) dense. Figs. 2 and 4 both involve the use of Zenker's fixative.
Fig. 5. Treatment, 6 and 11.
Although the neckpiece (n.p.) has been moved from its normal position. Continuity between the neckpiece and the tail (t.) is shown.
Fig. 6. Treatment, 1 and 10.
With fixation by osmium tetroxide the tail (t.) and neckpiece (n.p.) have become flattened. A connection between the tail and the neckpiece is shown.
Fig. 7. Treatment, 1 and 10.
The neckpiece (n.p.) has been torn from its normal position. An irregular surface structure is indicated in the neckpiece.

Plate II

Fig. 1. Treatment, 8 and 10.
Fig. 1 is an enlarged portion of Fig. 2 and shows the winding of the tail sheath (s.) around the axial filament. Tail fibrils (f.) can also be seen.
Fig. 2. The sheath has split and shrunk so that a periodicity in the sheath-winding along the tail is shown with areas in which the fibrils are visible (A) alternating with areas covered by the sheath (B).
Fig. 3. Fig. 3 is an enlarged portion of Fig. 2 and shows the winding of the tail sheath (s.) around the axial filament.
Fig. 4. Treatment, 4 and 11.
The neckpiece (n.p.) exhibits an irregular surface structure and is markedly flattened. The tail sheath (s.) loosely surrounds the axial filament.
Fig. 5. Treatment, 5 and 10.
The tip of the tail has split. The M fibrils lie in the center of a tube formed by the other fibrils.
Fig. 6. Treatment, 1 and 10.
The neckpiece (n.p.) and the tail (t.) are markedly flattened and fused to each other.

PLATE III

Fig. 1. Treatment, 9 and 12.
In the disrupted portion of an axial filament 10 fibrils are shown. One of the fibrils (M) is of greater diameter than are the others and is probably composed of two M fibrils.
Fig. 2.Treatment, 8 and 10.
The extension of the tail sheath (s.) beyond the tips of the L_1 fibrils is shown.
Fig. 3. Treatment, 9 and 12.
In Fig. 3 a the tips of the M fibrils (m.) have been disrupted to form a vesicle. In 3 b the tail sheath (s.) extend beyond the L_1 fibril tips to surround the M fibrils.
Fig. 4. Treatment, 9 and 12.
An ill-defined period is present in one fibril (L) in this disrupted area of an axial filament.
Fig. 5. Treatment, 9 and 12.
The tail sheath (s.) has been disrupted and lies on each side of the axial filament (a.f.) except for a short portion which adheres to the axial filament.
Fig. 6. Treatment, 9 and 12.
Proof that the tail sheath (s.) is a membrane and not a drying artefact is indicated. The sheath can be seen lying across the axial filament (a.f.). A drying artefact would not cross the axial filament in this manner.
Fig. 7. Treatment, 9 and 12.
An ill-defined period within the fibrils is shown in this disrupted portion of an axial filament.

PLATE IV

Fig. 1. Treatment, 1 and 10.
Flattening of the head and tail is shown. The tail (t.) is of constant width from its origin to the tips of the L fibrils (L) where it is reduced. At the tips of the L_1 fibrils (L_1) it is again reduced. The M fibrils continue to the tip of the tail.
Fig. 2. Treatment, 5 and 10.
The axial filament (a.f.) has been split and the M fibrils exposed.
Fig. 3. Treatment, 3 and 10.
In this disrupted portion of the axial filament the fibrils have become separated and exhibit a discontinuous and irregular period.

PLATE V

Fig. 1. Treatment 3 and 10.
The tail sheath has burst at a sharp bend with the fibrils (f.) forming a fanlike configuration.
Fig. 2. Treatment, 2 and 10.
The tail has been disrupted along most of its length with the fibrils (f.) becoming free. The neckpiece (n.p.) has been torn from its normal position and appears to be connected to 4 fibrils (F) of the axial filament.
Figs. 3 and 4. Treatment, 2 and 10.
Disruption of the tails of the spermotozoa with spreading of the fibrils at sharp bends is shown.
Fig. 5. Treatment, 1 and 12.
The tail fibrils (f.) have been spread and partially disrupted into subunits. The flattening of the structures precludes detection of the fine details within the fibrils.

PLATE VI

All figures: Treatment, 9 and 12.
Fig. 1. Protofibrils (p.) are shown within the M fibrils in the tip of the axial filament. The inset is an enlarged portion of an M fibril and shows two protofibrils.
Fig. 2. One of the M fibrils has been disrupted in area D. Unravelling of an L_1 fibril into its component protofibrils (p.) is also shown.
Fig. 3. Two protofibrils have coalesced at C. If this were to occur over an appreciable length of the fibril, a periodicity within the fibril would become apparent. In the upper M and L_1 fibrils protofibrils (p.) are shown.
Fig. 4. A vesicle has been formed at the ends of the M fibrils. Protofibrils are visible in both M fibrils and in one of the L_1 fibrils. At point U the protofibrils of the M fibril have unravelled.

PLATE VII

All figures: Treatment, 9 and 12.

Fig. 1. Protofibrils (p.) are shown in both the L_1 and M fibrils. At points U unravelling of the profibrils has occurred.

Fig. 2. In Fig. 2a a vesicle has formed at the tip of one of the L_1 fibrils. Unravelling of the protofibrils is shown in Figs. 2a and 2b. Coiling of protofibrils is shown within the fibrils in Figs. 2a, 2b, and 2c.

PLATE VIII

Treatment, 9 and 12.

The end of the L_1 fibril on the right has been broken and pulled away from the main portion of that structure. Within the end of this fibril the protofibrils (p.) are shown. With lesser clarity the protofibrils (p.) are shown in the other L_1 fibril. The protofibrils of the M fibrils have unravelled (U) and one of them (p.) is indicated within the vesicle. The remaining fibril (L) is probably an L fibril which has been stretched beyond the ends of the other L fibrils.

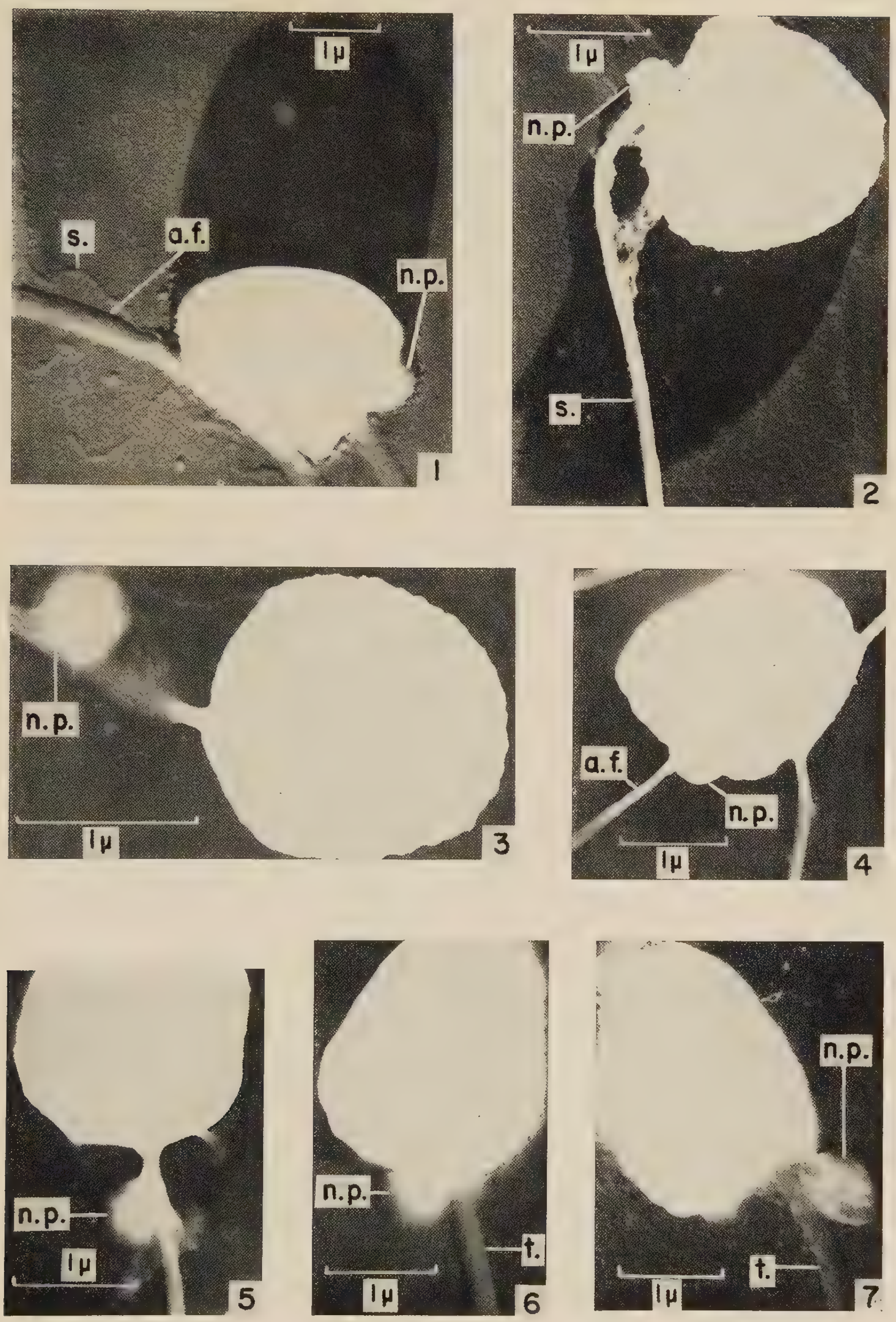

1μ
s.
a.f.
n.p.
1
1μ
n.p.
s.
2
n.p.
1μ
3
a.f.
n.p.
1μ
4
n.p.
1μ
5
n.p.
t.
1μ
6
n.p.
t.
1μ
7

PLATE I

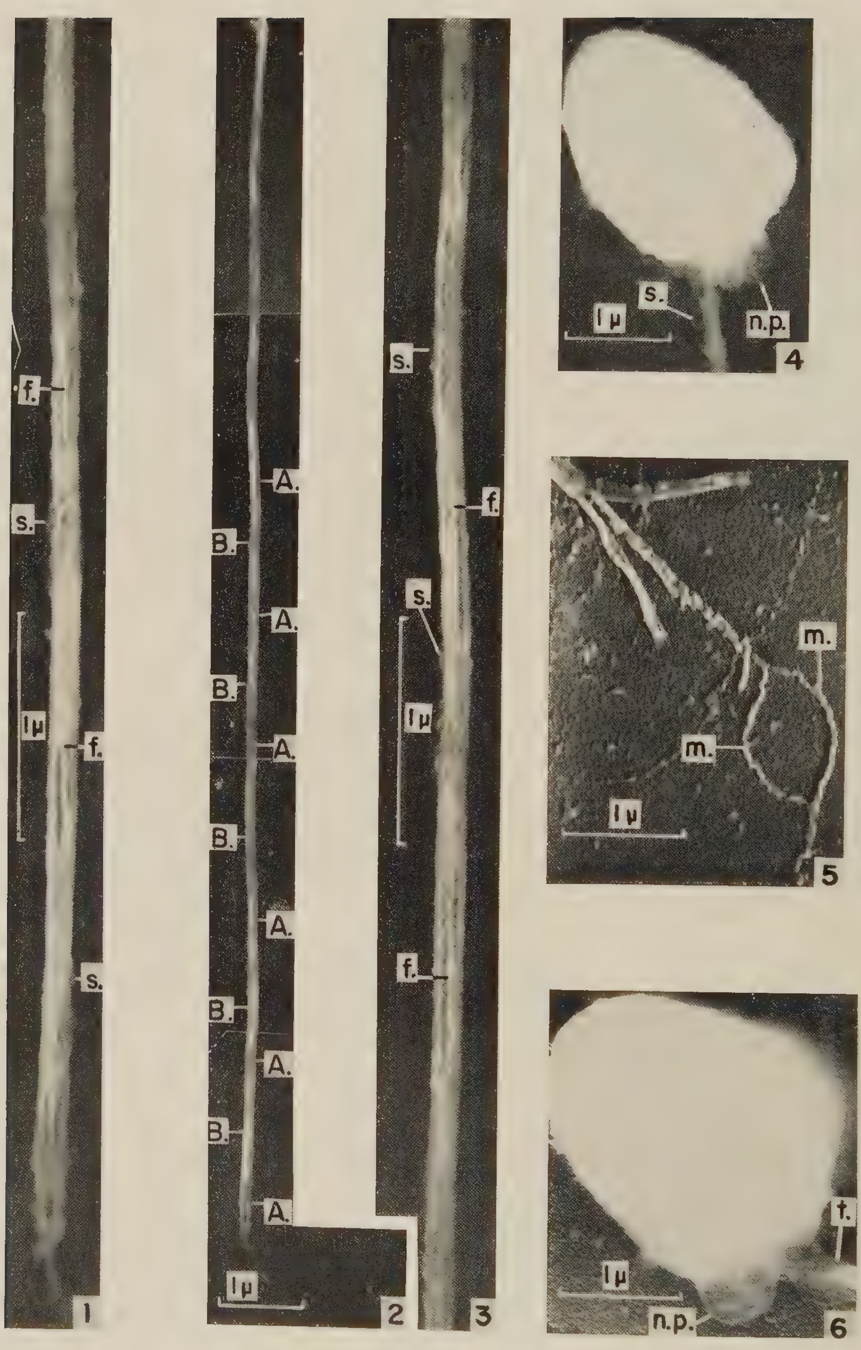
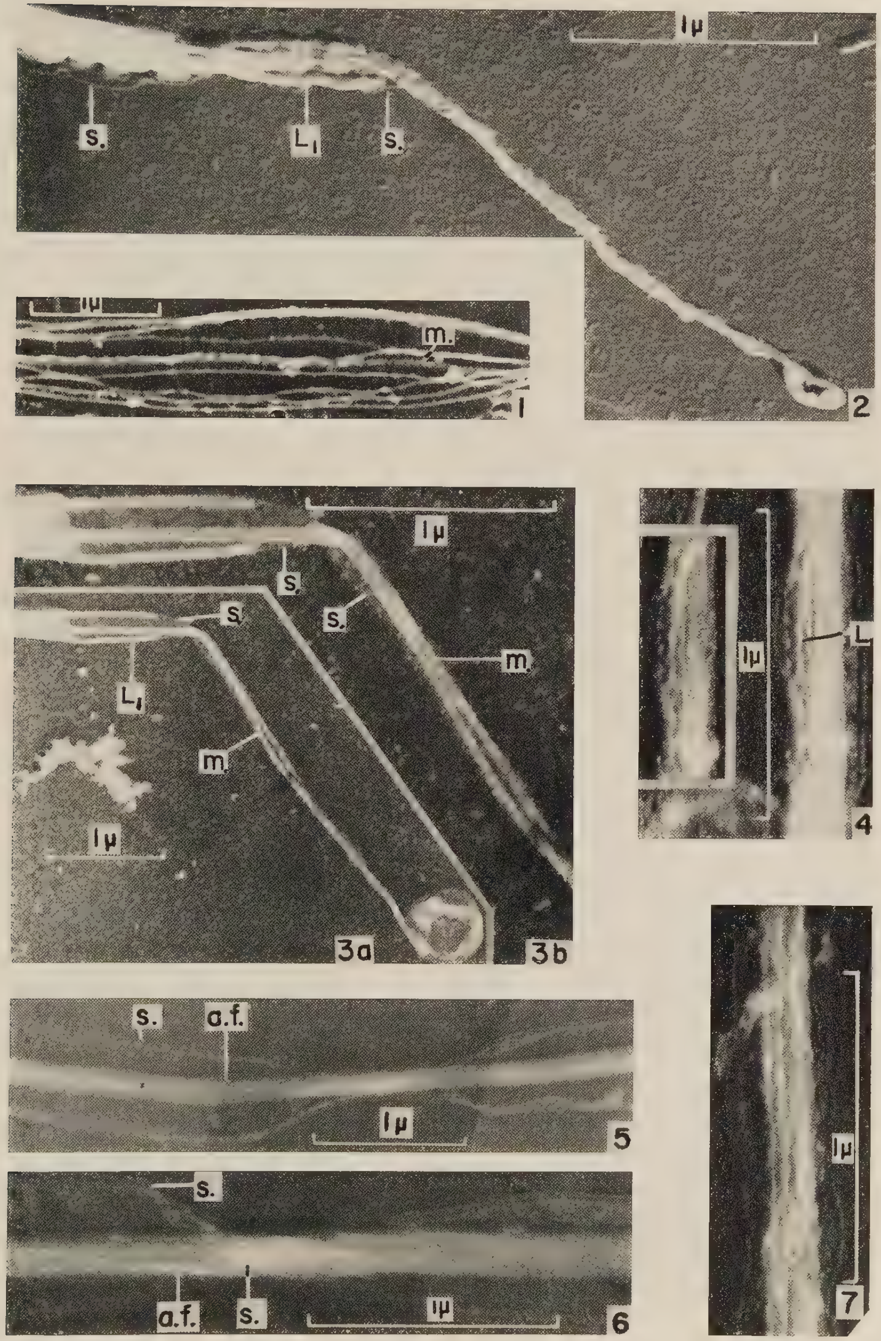

f.
s.
1µ
f.
s.
1
A.
B.
A.
B.
A.
B.
A.
B.
A.
B.
A.
1µ
2
s.
f.
s.
1µ
f.
3
s.
1µ
n.p.
4
m.
m.
1µ
5
t.
1µ
n.p.
6

PLATE II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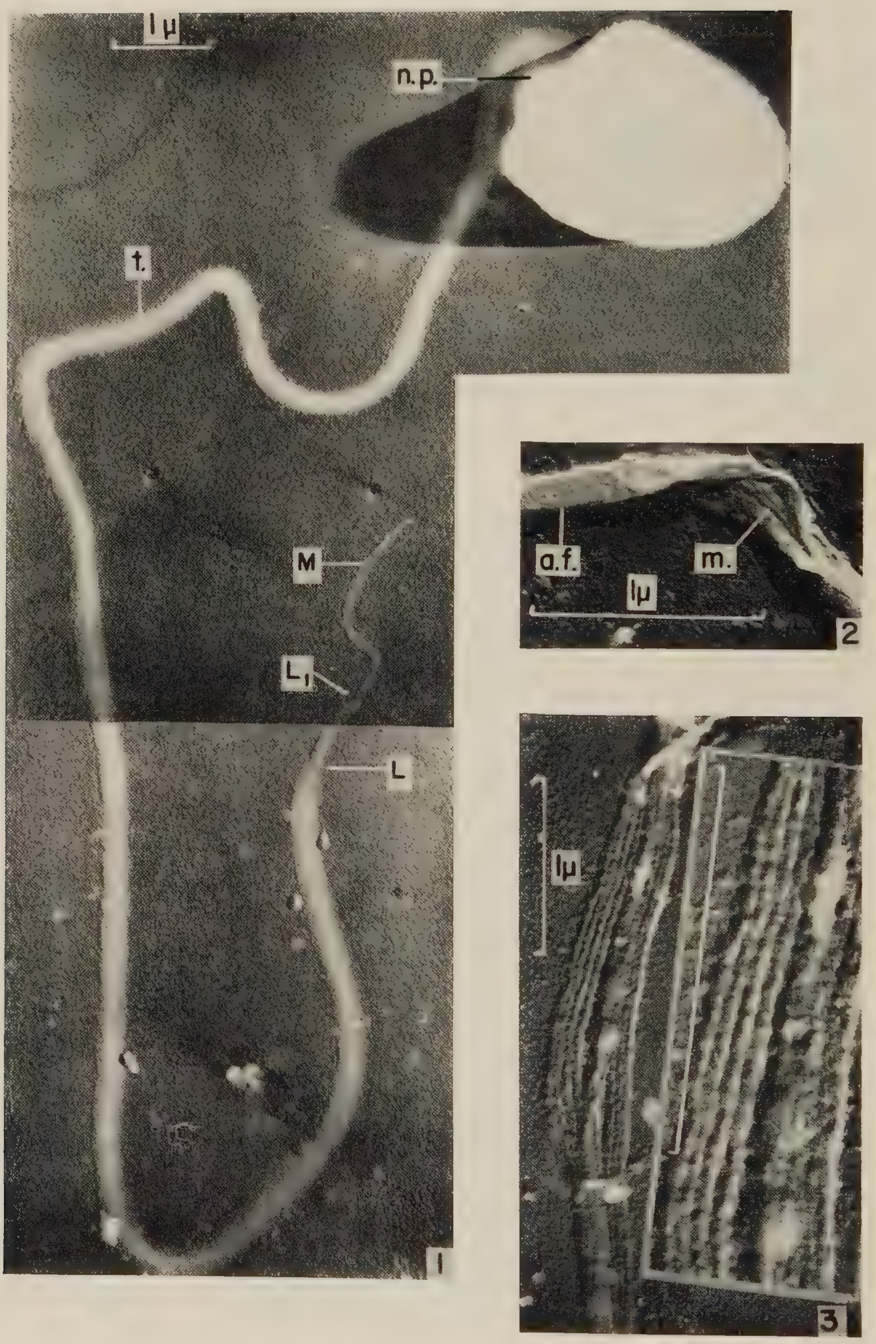
S.
L₁
S.
2
1μ
m.
1
1μ
S.
S.
S.
m.
L₁
m.
1μ
3a
3b
1μ
L
4
S.
a.f.
1μ
5
S.
a.f.
S.
1μ
6
1μ
7

Plate III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t.
M
L₁
L
1
a.f.
m.
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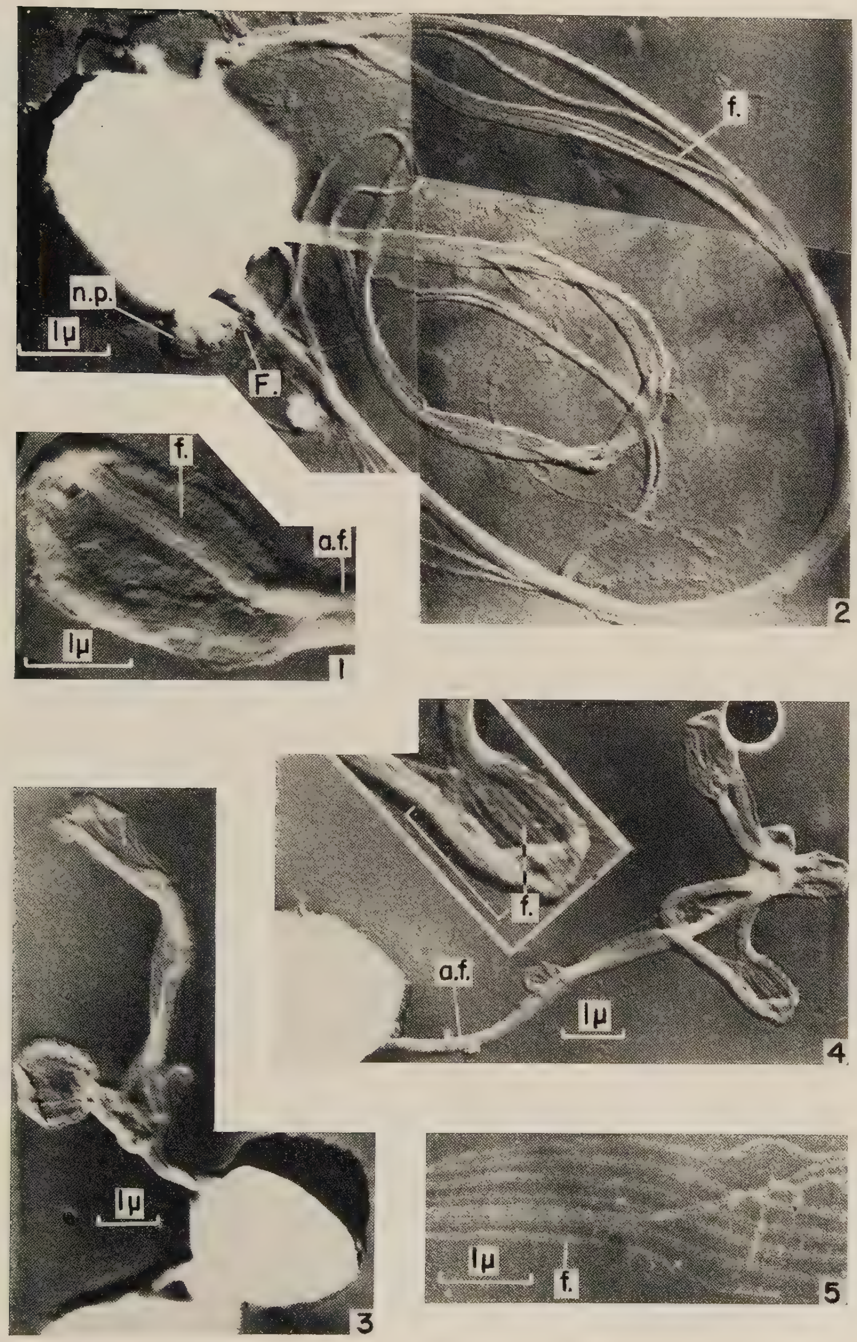
2
1μ
3

Plate IV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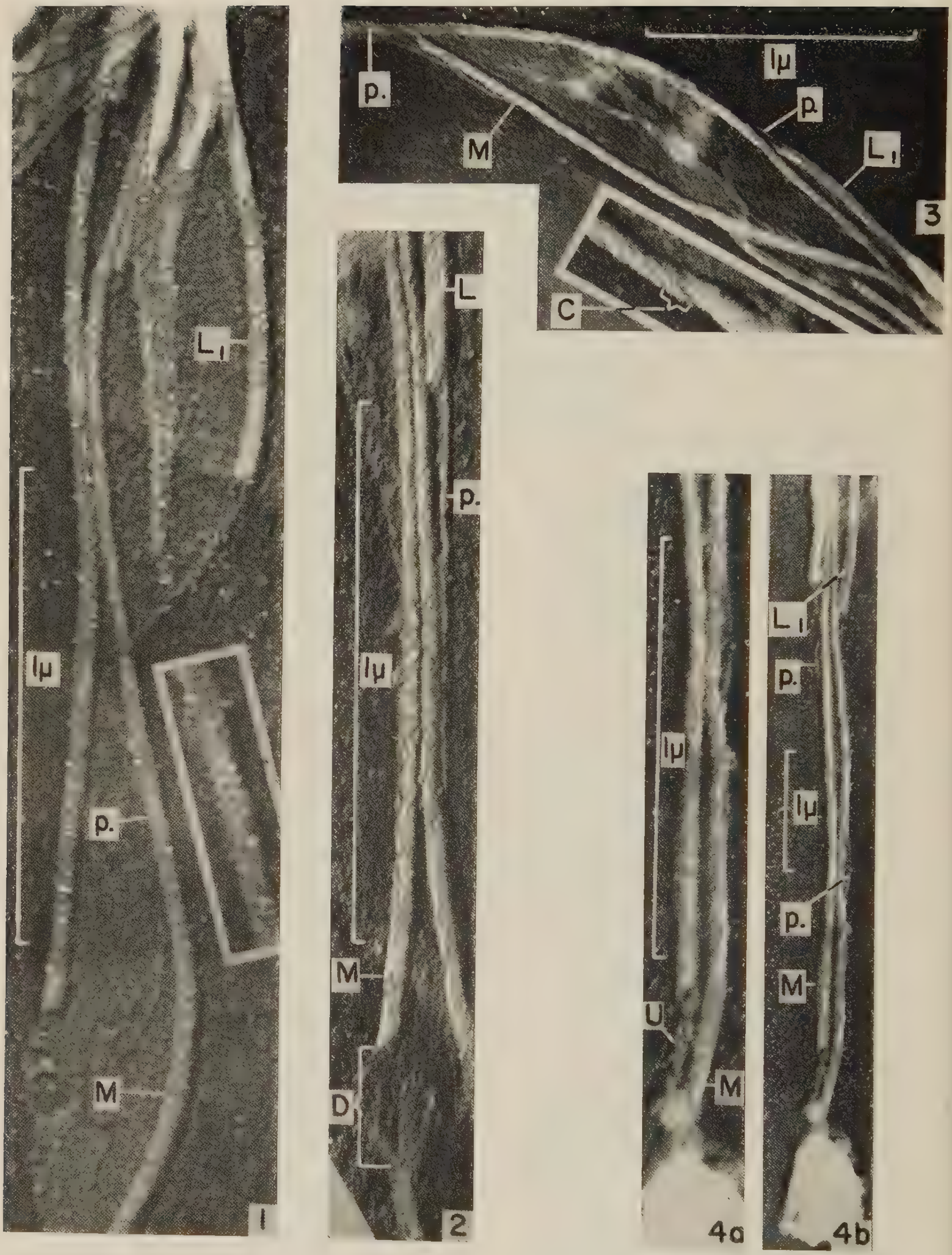
n.p.
1μ
F.
f.
a.f.
1μ
1
2
1μ
3
f.
a.f.
1μ
4
1μ
f.
5

PLATE V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L_1
p.
M
1
L
p.
1μ
M
D
2
p.
M
1μ
p.
L_1
C
3
1μ
U
M
4a
L_1
p.
1μ
p.
M
4b

Plate VI

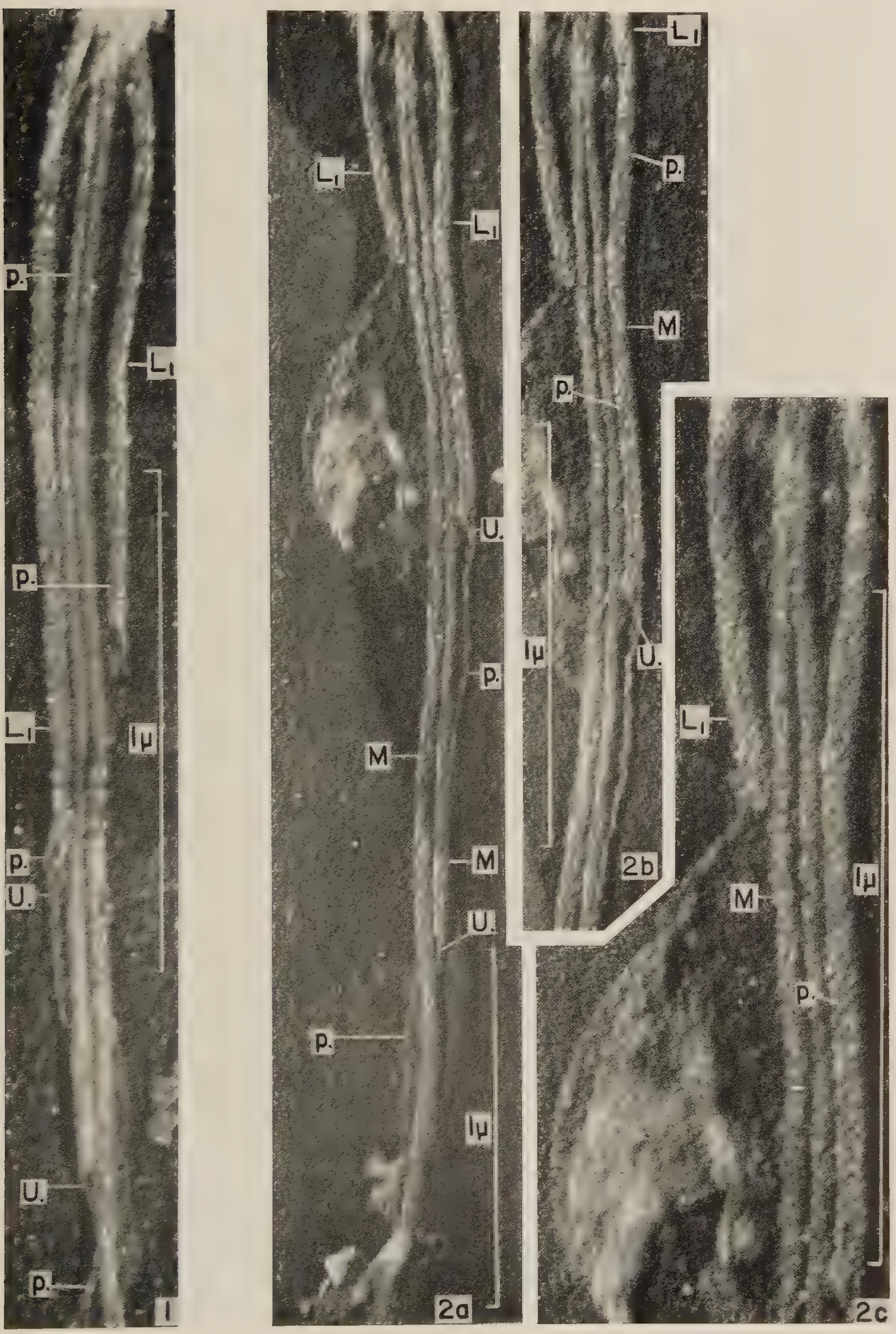
p.
L₁
p.
L₁
1μ
p.
U.
U.
p.
1
L₁
L₁
U.
p.
M
M
U.
p.
1μ
2a
L₁
p.
M
p.
1μ
U.
2b
L₁
M
p.
1μ
2c

PLATE VII

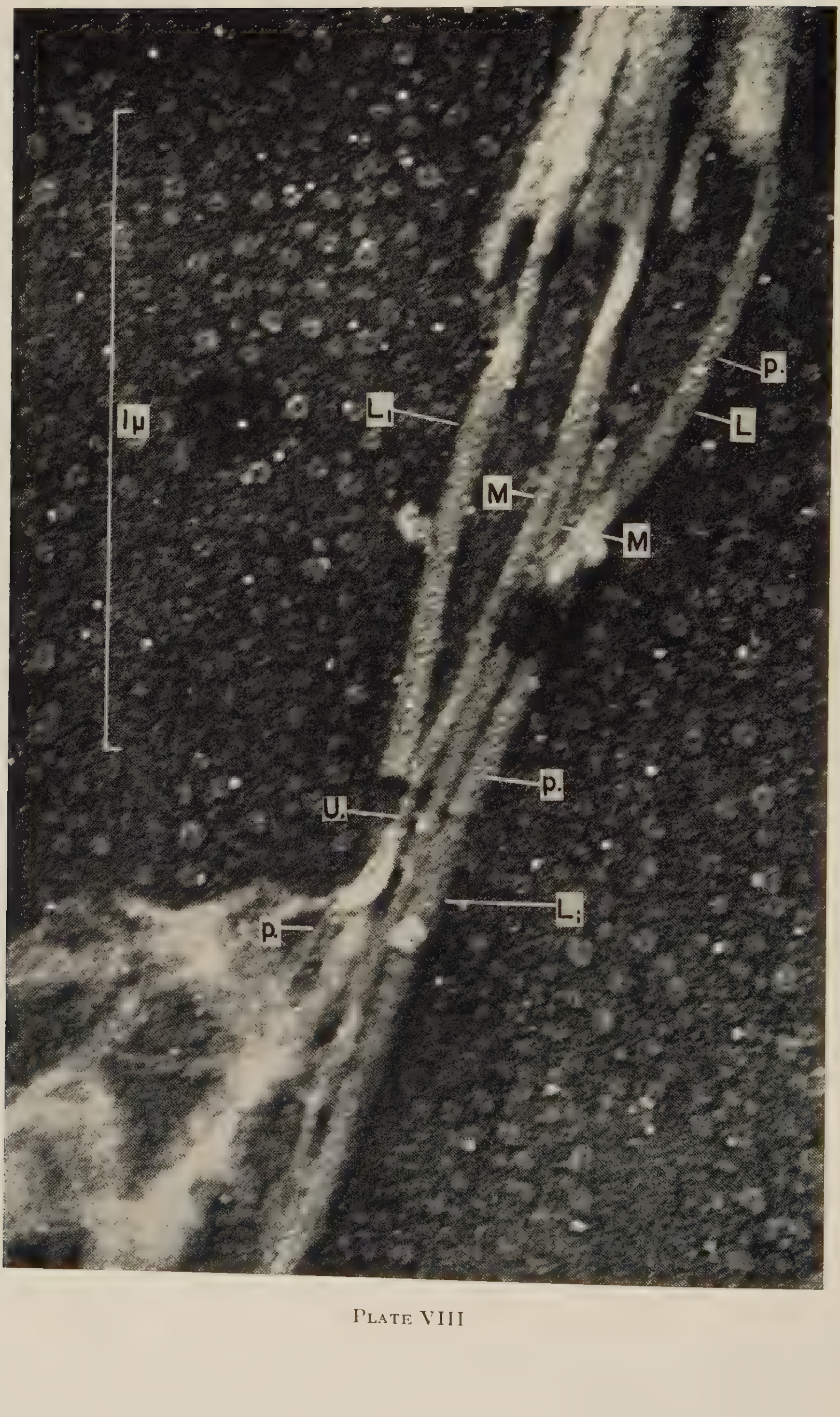
1μ
L₁
p.
L
M
M
p.
U
L₁
p.

PLATE VIII

STUDIES ON ISOLATED CELL COMPONENTS

VI. THE EFFECTS OF NUCLEASES AND PROTEASES ON RAT LIVER NUCLEI[1, 2]

N. G. ANDERSON[3]

Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn., and Department of Zoology, Duke University, Durham, North Carolina, U.S.A.

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The studies to be described, which concern the effects of several enzymes on fresh isolated rat liver nuclei (45) at pH 7.1, were initiated with the following two points in mind. First, it was thought that if nucleases and proteases had characteristic effects on the appearance of isolated nuclei, then the progressive changes observed in nuclei in whole homogenates might be ascribed to specific types of enzymes. Second, it appeared that certain problems of nuclear structure might be attacked through a study of specific enzyme action. Among these are the problems of the origin, structure, and permeability of the nuclear envelope, as well as certain problems concerning the colloidal-chemical properties of the nuclear substance and the nucleolus.

The experiments recorded bear little relation to the use of similar enzymes in histochemical procedures because a) no attempt has been made to remove the enzymatic substrate completely, b) the effects of interest occur in a matter of minutes instead of hours, and c) the reactions involve fresh, unfixed materials, presumably in a native state.

While the effects of nucleases on *Drosophila* salivary gland chromosomes have been studied by a number of workers (9, 26, 32, 34, 35, 36, 38, 42) the results are not comparable to those obtained here, since different pretreatments were used. Studies on the morphological changes induced in whole fresh nuclei by various enzymes do not appear to have been made. Barton (6) showed that a certain fraction of the desoxyribonucleic acid (DNA) of isolated nuclei was not removed by prolonged treatment with desoxyribo-

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[1] Work at Oak Ridge performed under Contract No. W-7405-Eng-26 for the Atomic Energy Commission.

[2] A brief account of this work was presented before the Association of Southeastern Biologists in April 1952. (J. Tenn. Acad. Sci., **27**, 198, 1952.)

[3] Atomic Energy Commission Postdoctoral Fellow.

nuclease (DNAase) while Laskowski and Engbring (28) used nuclei treated with DNAase in a study of nuclear proteins. In the latter two studies no microscopical observations were reported, however.

METHODS

Rat liver nuclei were isolated in a phosphate-bicarbonate buffer containing sucrose essentially as previously described (45). Both Osborne-Mendel and Sprague-Dawley strains of rats were used. Emphasis was placed on rapidity of preparation rather than purity, and nuclei were rarely observed longer than 2½ hours after killing the rat. Nuclei were prepared and maintained at 0° C but observations and phase contrast photomicrographs were made at room temperature. All solutions were made up in pyrex double distilled water. Crystalline ribonuclease (RNAase), DNAase, chymotrypsin, and twice crystallized salt-free trypsin were obtained from the Worthington Biochemical Company. Enzyme solutions were made up in the isolation medium (Solution I).[1] The effects observed with nucleases were confirmed with enzyme solutions dialyzed against Solution I in the cold to insure complete removal of divalent cations which have been previously shown to have a marked effect on the size and appearance of the isolated rat liver nucleus (4).

RESULTS

Desoxyribonuclease. When DNAase (1 mg/ml Solution I) was introduced under the cover slip the nuclei gradually assumed an empty appearance (Figs. 1, 2), and the nucleoli appeared to condense into irregular masses. The nuclear envelope (karyotheca), much more distinctly seen after this treatment, was rather thick. The clearing process required about 1–4 minutes at room temperatures and appeared to take place evenly throughout the nuclei. Two general types of nuclei could be distinguished at the end of this time in the same preparation. The most predominant type was characterized by free Brownian movement of the nucleoli and by the presence of variable numbers of free granules in very rapid Brownian movement (Fig. 4). A few granules were sometimes seen along the inner edge of the nuclei in this type. The second type showed little if any nucleolar Brownian movement, while the main body of the nucleus was filled with a very fine reticulation (Fig. 3). The difference between the two types may be chiefly one of degree, since the fine reticulation of the first type was often seen to condense gradually into predominantly rod-shaped structures, some of which became attached to the inner surface of the nuclear envelope. In some nuclei, however, very few granules were seen and these were too small to determine whether they had a characteristic shape. DNAase treatment also caused the appearance of occasional blebs on the nuclear surface.

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[1] The following solutions have been used for isolating nuclei in the present series of studies: *Solution I.* 0.0094 *M* KH_2PO_4, 0.0125 *M* K_2HPO_4, 0.0015 *M* $NaHCO_3$, 0.145 *M* sucrose. *Solution II.* Same salt concentration as Solution I, sucrose 0.218 *M*. *Solution III.* Same salt concentration as Solution I, sucrose 0.272 *M*.

The effects of DNAase may be readily demonstrated using lower concentrations of the enzyme. Thus a solution containing 0.01 mg of DNAase/ml (prepared immediately before use) was observed to begin to clear the nuclei in about 10 minutes. Since no divalent cations were added in these experiments, it is evident that the DNAase was not optimally activated.

Nuclei observed with a high, dry, dark contrast phase objective appeared as dark spheres against a grey background. DNAase caused a rapid loss of this apparent optical density (Figs. 5, 6). With oil immersion dark contrast phase objectives, however, the intact nuclei appear very little darker than the background; therefore, the clearing effect of DNAase does not result in as marked a change in appearance (Figs. 12–15).

While DNAase-treated nuclei appear virtually empty in many instances, the addition of basic dyes (Janus green B, methylene blue, pyronin) after DNAse was found to precipitate a considerable amount of material in the nucleus (Figs. 9, 10). No methyl green staining material was evident in the nucleus after DNAase treatment except for a slight staining of the nucleoli.

Distilled water or salt-free sucrose solutions after DNAase treatment caused the nucleoli and the residual nuclear material to swell rapidly and disappear (Figs. 5–7). Little basic staining material was demonstrable within the nuclear envelope after this treatment (Fig. 8). More prolonged treatment with distilled water resulted in extreme bleb formation exceeding that previously observed in 0.001 *M* magnesium chloride (2). Except for the formation of blebs, the nuclear envelope remained dense and intact during the treatment described.

When nuclei previously shrunk with 0.001 *M* magnesium chloride were treated with DNAase made up in the same solution, no change in appearance was noted. However, the nuclei failed to swell and regain a nongranular appearance when replaced in Solution I, indicating that the DNA gel had been depolymerized. (Evidence relating the effect of salts on nuclei to polymerization of the DNA has been presented in a previous paper in this series (4)).

Nuclei isolated by the technique used here have a marked tendency to stick together (45). DNAase was found to decrease this stickiness.

Ribonuclease. Ribonuclease (0.001–1.0 mg/ml in Solution I) caused the nucleoli to condense into dark irregular masses (Figs. 11, 13). Other condensations varying in number and density were evident throughout the rest of the nuclear mass. No change in the nuclear envelope was noted, and no general clearing of the nuclear substance such as occurred with DNAase was seen. Nucleoli condensed by RNAase treatment could be dispersed with distilled water, sucrose solutions free of salt, or by heparin in Solution I.

RNAase solutions heated to 100° C for 3 minutes in sealed tubes were found to give the same results as unheated preparations, demonstrating that the effects observed were not due to contamination with thermolabile enzymes.

Desoxyribonuclease followed by ribonuclease. Nuclei treated with DNAase and then exposed to RNAase differ in their response. Many appeared virtually empty except for the nucleoli which often appeared as hollow structures; others exhibited many small granules in rapid Brownian movement. Blebs were also frequently seen.

Ribonuclease followed by desoxyribonuclease. Treatment with DNAase subsequent to RNAase resulted in clearing of the nuclei as seen under the phase microscope, and the appearance of many small granules in rapid Brownian movement (Figs. 12-15) which did not appear to originate in the nucleoli. The number of these granules was very variable, ranging from many and including rods and irregular shapes (Fig. 16) to almost none. These differences were often seen among adjacent nuclei on the same slide. In the photographs shown it should be noted that many small granules present cannot be seen because of Brownian movement during exposure.

Trypsin. Trypsin (0.01–1.0 mg/ml in Solution I) caused a rapid decrease in nuclear density, due evidently to the extrusion of a DNA-containing gel (33), which was observed to push the nuclei apart. With high concentrations of trypsin (1 mg/ml) rupture and partial disintegration of the karyotheca was observed to take place in a few seconds (Figs. 17, 18). The nucleoli disappeared almost at once. Trypsin also digested away the granular contents remaining in nuclei treated with RNAase and DNAase. In preparations from Osborn-Mendel rats the nuclear envelopes remained intact and spherical after this treatment, but were very much thinner (Figs. 19, 20). They resembled the nuclear blebs previously described (2). With Sprague-Dawley liver nuclei, nuclease treatment followed by trypsin left residual envelopes which resembled hollow myelin figures, did not hold their shape, and tended to coalesce to form irregular masses.

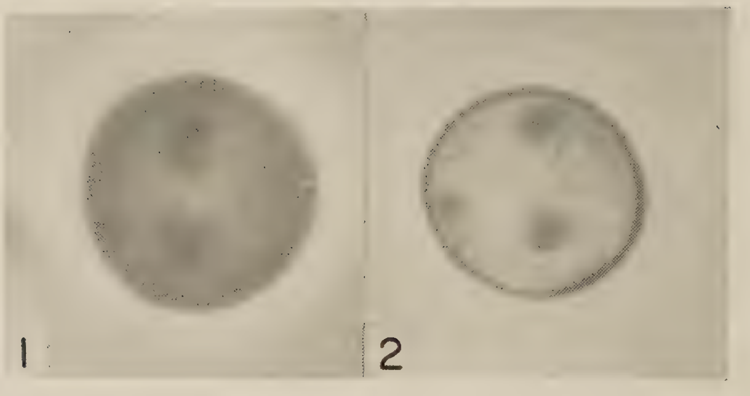
Nuclei shrunken with 0.01 M magnesium chloride and then treated with trypsin made up in the same solution (1 mg/ml) showed little immediate change. Very gradually the nuclei became less "brilliant" and many oblong bodies appeared in them. These results are in marked contrast to those obtained with trypsin in Solution I where rapid dissolution took place.

Chymotrypsin. Results essentially similar to those described for trypsin were obtained.

Observations on nuclei in aged homogenates. Nuclei in homogenates (1 g liver/20 ml homogenate) at pH 7.1 gradually lost their density, as observed under the phase contrast microscope. The nucleoli disappeared and the nuclei shrank somewhat. The karyotheca remained intact but much thinner. In homogenates incubated at 37° C changes were first detectable in about 30 minutes and consisted either in clearing or in a slight granulation. Few intact nuclei could be found after 2 hours. When homogenates were left at 0° C, however, little change was observed for several hours. After 18 hours many nuclei were still intact while others were almost empty with no internal structure. In all instances a very considerable difference was evident in the degree of clearing of different nuclei in the same preparation.

DISCUSSION

The effects of DNAase, RNAase, and proteolytic enzymes (trypsin and chymotrypsin) have been shown to be characteristic and distinct. With DNAase the gel-like structure of the nucleus was rapidly broken down with a consequent loss of physical density and image density as observed under the dark contrast phase microscope. (The relation between image density and object refractive index in biological materials with the phase microscope has been discussed by Crawford and Barer (12)). Brownian movement of the nucleoli and granules which generally condensed in the nuclei gave further evidence of loss of gel structure. RNAase, in contrast, caused the nucleoli to condense into small dark masses, but caused no general clearing of the nuclei. The proteolytic enzymes trypsin and chymotrypsin both caused rapid disintegration of the nuclei leaving only shreds of the ruptured nuclear envelopes. These effects are sufficiently distinct to allow tentative identification of the types of enzymes responsible for the alterations observed in aged homogenates. The general clearing of the nucleus at pH 7.1 with a slight thinning of the nuclear envelope appears to be due to both proteolytic activity and DNAase. It is thought that the proteases account for the lack of internal structure (granules and nucleoli), while DNA is lost by a combination of DNAase depolymerization and protease displacement. The proteolytic activity of the homogenate is also thought to account for the thinning of the envelope. It should be noted that no increase in homogenate viscosity has been



Figs. 1 and 2. Isolated rat liver nucleus in Solution I, and 5 minutes after exposure to desoxyribonuclease (1 mg/ml in Solution I). Note general clearing and nucleolar condensation. Photographed with 4-mm dark contrast medium objective.

found with ageing, indicating that the DNA is not merely displaced but is also depolymerized. Marked loss of DNA from nuclei in incubated homogenates has been previously observed (3).

Recently it has been shown that tissue DNAase is almost inactive above pH 7.0 and does not require divalent cations for activation (44). The pancreatic DNAase used here differs from tissue DNAase in having a broad pH optimum between 6 and 7, and in requiring divalent cations for maximal activation (27). While tissue DNAase and pancreatic DNAase differ in these two properties, it is assumed that their effects on nuclear structure are similar. The fact that pancreatic DNAase attacks isolated nuclei readily in solutions free of divalent cations suggests that these ions are present in the isolated nucleus. This view is supported by the finding that nucleohistone isolated from calf thymus contains an appreciable amount of magnesium (21).

Results obtained with various enzyme preparations such as are reported here cannot be evaluated properly unless something is known of their purity

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All photographs made with dark contrast medium phase contrast objectives.

Fig. 3. Isolated rat liver nuclei 6 minutes after DNAase (1 mg/ml in Solution I). 4-mm objective.

Fig. 4. Isolated rat liver nuclei from same preparation shown in Fig. 3, $3\frac{1}{2}$ minutes after DNAase (1mg/ml in Solution I). Notice difference in internal granulation and wrinkling of envelopes.

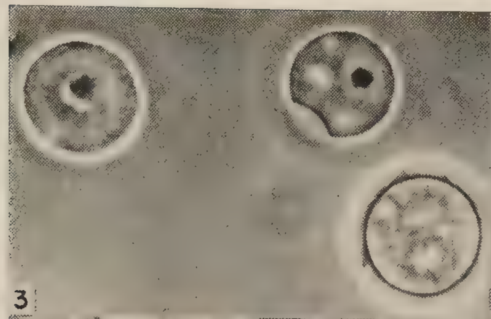
Fig. 5. Isolated rat liver nuclei in Solution I.

Fig. 6. Same nuclei 5 minutes after DNAase (1 mg/ml in Solution I).

Fig. 7. Same nuclei 5 minutes after 0.88 *M* sucrose.

Fig. 8. Same nuclei after pyronin-methyl green in Solution I. Figs. 5–8 photographed with 4-mm objective.

Figs. 9 and 10 Nucleus treated with DNAase and then basic dyes (pyronin and crystal violet in Solution I). Note blebs in Fig. 10.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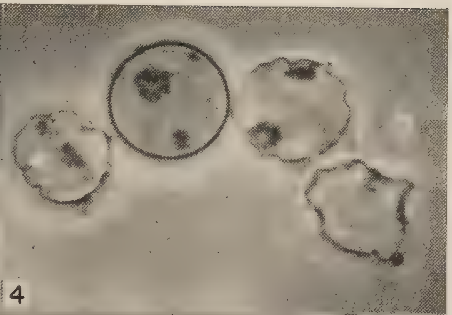
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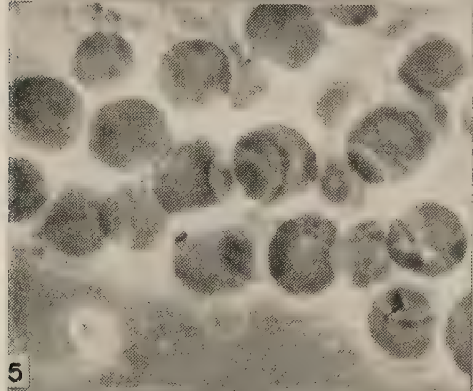
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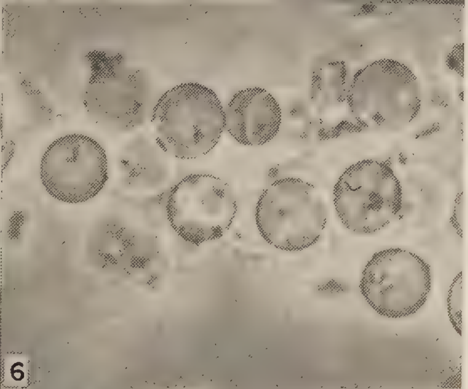
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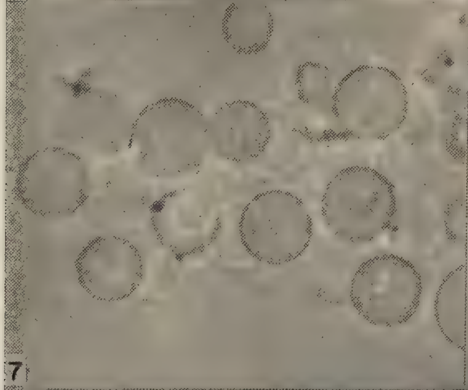
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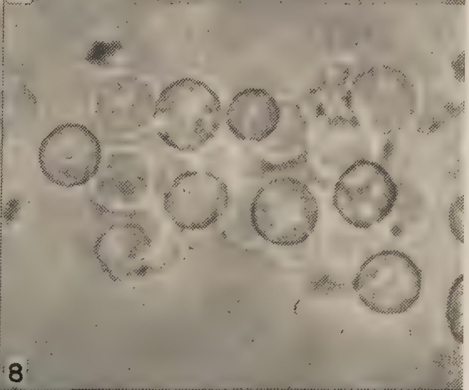
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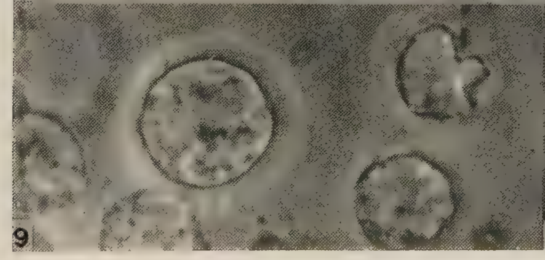
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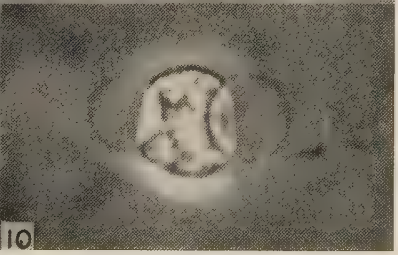
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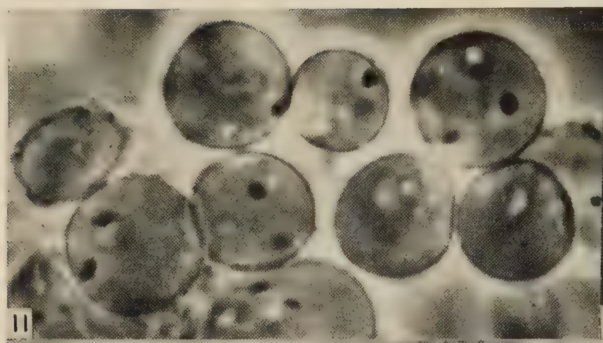
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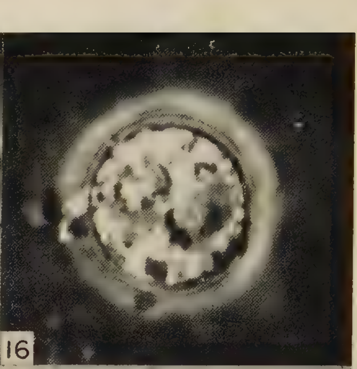
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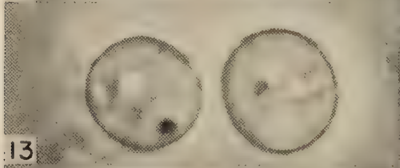
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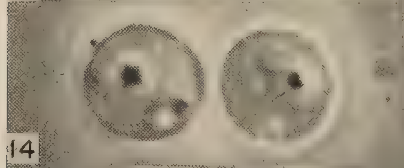
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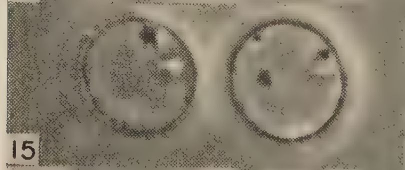
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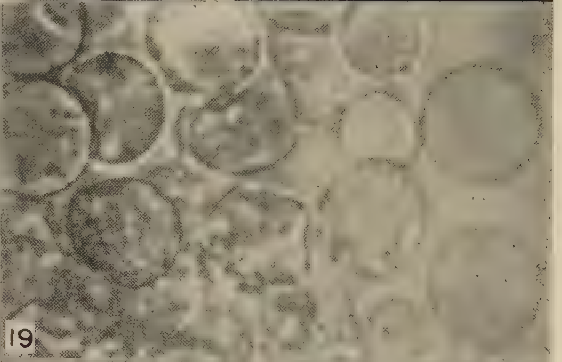
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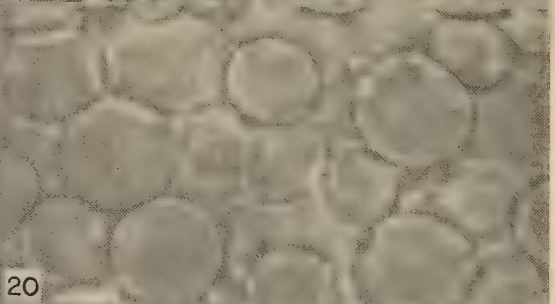
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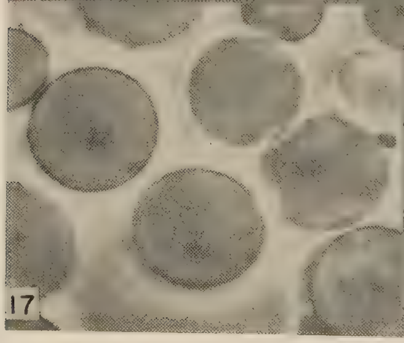
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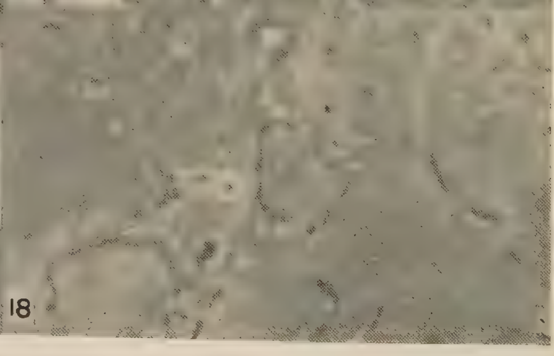
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and specificity. In this regard it should be noted that much attention has been given to proteolytic contaminants found to be present in nucleases used by earlier workers in the field (see (25)). Some doubt also appears to have existed regarding the specificity of the two nucleases generally used. Recent work, however, shows that DNAase and RNAase prepared according to accepted methods are specific for DNA and RNA respectively, and have no measurable nonspecific nuclease activity (16).

The effects of DNAase and RNAase reported here are not believed to have been influenced by possible proteolytic contamination for the following four reasons: 1) The results obtained with RNAase have been confirmed using an enzyme preparation which had been previously heated to 100° C. This destroys the activity of proteolytic enzymes but does not irreversibly inactivate ribonuclease (15, 20, 43). 2) Preparations obtained from the same sources as those used here have generally been found to be free of measurable proteolytic activity (E. Volkin, personal communication). 3) In the type of study where proteolytic contamination has been a matter of some concern, histological sections have been exposed to the enzymes at relatively high temperatures (usually 37° C) for periods of time varying from 1–2 hours up to 12 hours. Enzyme concentrations have usually varied from 0.01–1.0 mg/ml for DNAase and 0.1–6.0 mg/ml for RNAase (see 25). In the present study the effects reported have been observed to occur in 30 seconds with RNAase and 1–4 minutes with DNAase (using concentrations of 1 mg/ml) at room temperature. Proteolytic contaminants exerting slight effect over a prolonged period at 37° C would be expected to have little effect on reactions observed to take place in seconds or minutes at room temperature. 4) In the studies reported here, crystalline trypsin has been shown to produce

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Fig. 11. Isolated rat liver nuclei 2 minutes after exposure to ribonuclease (1 mg/ml in Solution I).
Fig. 12. Nuclei in Solution I.
Figs. 13 and 14. Same nuclei 2 and 3 minutes after RNAase (1 mg/ml in Solution I). Focused at different levels. Note nucleolar condensation and slight evidence of structure throughout body of nuclei.
Fig. 15. Same nuclei 4 minutes after DNAase in Solution I. Brownian movement of the nucleoli was evident. Many small granules in rapid Brownian movement are not visible on the photograph. Photographed with oil immersion phase contrast objective. Note difference in appearance of nuclei with this lens and with 4-mm phase objective (Figs. 1 and 5).
Fig. 16. Nucleus treated with RNAase followed by DNAase. Note many internal condensations which were in rapid Brownian movement.
Figs. 17 and 18. Rat liver nuclei before and 2 minutes after trypsin (1 mg/ml in Solution I). Nucleoli disappear very rapidly, nuclear gel expands, and nuclear envelope ruptures. Fragments of the envelope persist.
Fig. 19. Nuclei treated with DNAase and RNAase in the same solution and then exposed to trypsin (0.1 mg/ml in Solution I). Trypsin solution moving in from right side digests residual nuclear contents and leaves much thinned envelope intact.
Fig. 20. Nuclei treated as in Fig. 19, 10 minutes after introduction of the trypsin.

effects on the isolated nucleus which stand in marked contrast to the results obtained with nucleases. Trypsin digested and removed rapidly and completely the granules and condensed nucleoli which appeared after RNAase and DNAase treatment, while prolonged exposure to either nuclease did not have this effect. Trypsin also digested away most of the nuclear envelope, leaving only a very thin residue. RNAase and DNAase either had no effect on the envelope or made it appear thicker. It is concluded that the effects reported are due to the specific enzymes used and are not affected by contamination with other enzymes or, since dialyzed preparations gave identical results, by extraneous salts.

The possibility must be considered, however, that some of the effects are not enzymatic in the usual meaning of the term. In previous studies (3) it has been shown that DNA may be displaced from the nucleus by the highly charged heparin molecule which combines with the basic nuclear proteins. The result of the formation of a trypsin-protein enzyme-substrate complex may be displacement of DNA even before actual digestion of the protein has begun. The rapidity of the release of DNA by trypsin has been noted by Mazia (33) who used trypsin to prepare highly polymerized DNA.

As previously pointed out by Callan (7), the problem of the permeability of the nuclear envelope is a central one in any consideration of nuclear-cytoplasmic interrelationships. It appears to be assumed generally that it is permeable to rather small molecules only (18) and that the nucleus is capable of exhibiting osmotic properties (10). The changes in nuclear volume which accompany alterations in ionic composition of the suspending medium and which are believed to account for the "pseudo-osmotic" behavior of somatic cell nuclei have been discussed previously (4), and it is not felt that any available data on nuclear volume changes have demonstrated rigorously the existence of a semipermeable nuclear membrane. Goldstein and Harding (17) have found that protein solutions could be devised which would prevent the swelling of isolated frog oöcyte nuclei. Such nuclei, which are not composed chiefly of hydrated chromosomes, as appears to be the case with animal somatic cell nuclei, and which have enlarged considerably by the accumulation of substances within the nucleus, may have very different properties from those of the nuclei studied here. Also, in protein solutions of any considerable concentration, the solution volume of the protein lowers the concentration of water below 55.5 M, resulting in a slight dehydrating effect, which may be sufficient to prevent swelling of the nuclear colloids in the absence of a semipermeable membrane.

Nuclei isolated in aqueous media have been shown to have a lower protein concentration per nucleus than either fixed cell nuclei (39) or nuclei isolated in nonaqueous media (1, 14). Also nuclei isolated by the latter method have been found to lose an appreciable amount of protein when placed in aqueous media. Highly polymerized DNA is extruded through the apparently undamaged envelope by heparin treatment (3), and in the present work, nucleases have been shown to penetrate rapidly. After DNAase and RNAase, distilled water or salt-free sucrose have been shown to disperse the residual intranuclear material, again without destroying the nuclear envelope. These observations show that the envelope of the isolated rat liver nucleus is readily permeable to proteins and other high molecular weight substances.

Two possibilities, however, prevent any final conclusion from being drawn from these observations. First, the nuclear envelope may well be altered by all isolation techniques thus far employed; and second, the substances demonstrated to penetrate the envelope (nucleases, heparin, etc.) may alter it themselves. Thus if the envelope contained patches of DNA and RNA, the nucleases might enter by destroying these substrate areas. At the present time, however, the observations cited, together with the studies of Caspersson (8) on nucleolar activity, and recent observations suggesting the nucleolar synthesis of RNA and its subsequent movement to the cytoplasm (5, 19, 31), all favor the conclusion that the nuclear envelope of the intact somatic cell is a porous, mesh-like structure. The small cytoplasmic particulates are not thought to penetrate it. The substance of the nucleus itself does not appear to impede significantly the free diffusion of dissolved enzymes. This is shown by the fact that DNAase digestion occurs evenly throughout the nuclei and not from the edge inward, and by the rapidity with which RNAase affects the nucleoli.

As previously noted (45) nuclei isolated at pH 7.1 have a marked tendency to stick together. The stickiness may be diminished by (a) lowering the pH (13), (b) by adding small amounts of divalent cations (4, 41), or (c) by treatment with DNAase. This suggested that an acidic substance is involved and that it is DNA. It does not appear unlikely that some strands of DNA may extend through the porous nuclear envelope and account, at least in part, for the clumping observed in the divalent cation-free isolation medium. Whether the stickiness observed is characteristic of the intracellular nucleus is not known. However the observation that nuclei of binucleate cells are generally in contact and tend to remain so (18) suggests that it is.

The nucleolar condensation produced by DNAase admits of no simple explanation. The apparent increase in phase contrast image density may be due to the removal of the surrounding dense DNA. The decrease in size may be the result of the digestion of the nucleolus-associated chromatin or to condensation of nucleolar RNA with histone freed from its association with DNA.[1]

Observations on the effects of distilled water on the nuclear envelopes remaining after RNAase-DNAase treatment suggest that bleb formation (2) is characteristic of the envelope itself. Further studies on the properties of this structure are in progress.

It has been suggested that the nuclear envelope is composed predominantly of basic proteins (10, 11). Hughes (18) has proposed that the nucleolus and the envelope may have a somewhat similar composition since they both tend to disappear at the same time during prophase. In the present work, however, several differences in the properties of these two structures have been demonstrated. The nucleolus has been shown to condense with nucleases, and to be dispersed by distilled water, salt-free sucrose solutions, heparin, trypsin, and chymotrypsin. The nuclear envelope, in contrast, has been found to show no comparable change after treatment with nucleases or heparin. Distilled water caused marked bleb formation but did not appear to remove substances from the envelope. Proteases digest the karyotheca rather slowly but cause almost immediate dissolution of the nucleoli. Further, in incubated homogenates the nucleoli were observed to disappear while the nuclear envelope was still intact and apparently unchanged. These observations suggest that the karyotheca is a rather stable structure, while the constituent substances of the nucleoli appear to be loosely bound.

It has been proposed that the nuclear envelope is composed of the peripheral sections of the chromosomal sheaths or vesicles (22, 23, 29, 30, 40). No evidence supporting this view has been obtained here. A number of procedures have been used which leave intact nuclear envelopes with no indication of internal septation. While the envelope may well originate from the chromosomal sheaths, it is evident that it differentiates in the interphase rat liver nucleus into a separate and distinct organelle.

The differences observed in nuclei in the same preparation after nuclease treatment or ageing may well reflect differences in the properties of nuclei at different times during interphase or in the physiological state of the individual cells.

The intranuclear granules observed after nuclease digestion may be comparable to the so called "residual chromosomes" (37). However, considering the variable size and number of these particles, this conclusion is not warranted at the present time. Also it does not appear profitable to attempt to relate in detail the results of the present investigation to previous studies on the effects of enzymes on giant chromosomes (9, 24, 26, 32, 35, 36, 38, 42), since fixatives or solutions of markedly different ionic composition were used.

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[1] The effects of basic proteins on nuclear structures have been investigated and will be discussed in a subsequent publication.

A more complete interpretation of the intranuclear structures produced by the treatments described will undoubtedly require the use of traditional cytological techniques.

SUMMARY

The effects of two nucleases and two proteases on isolated rat liver cell nuclei in a phosphate-bicarbonate-sucrose medium at pH 7.1 have been studied.

Desoxyribonuclease caused 1) rapid loss of density and a virtually empty appearance under the phase contrast microscope, 2) condensation of the nucleoli into granular masses which often exhibited Brownian movement, 3) a slight decrease in nuclear diameter, and 4) a sharper definition of the nuclear envelope. Brownian movement of intranuclear particles is taken as an indication of loss of gel structure (depolymerization). The effects were observed 1–4 minutes after adding the enzyme solution (1 mg/ml).

Ribonuclease (0.001–1.0 mg/ml) caused the nucleoli to condense into small dense masses within 30 seconds. A variable number of other very small condensations were also seen distributed throughout the body of the nucleus. No general clearing of the nuclear mass was detected and no Brownian movement of intranuclear particles was seen.

Treatment with both nucleases resulted in the formation of numerous intranuclear particles which, together with the condensed nucleoli, showed rapid Brownian movement.

Trypsin and chymotrypsin caused a rapid expansion of the nuclear gel and rupture of the nuclear envelope, which appeared much more resistant to digestion than nucleoli or intranuclear granules produced by nucleases.

The gradual clearing observed in incubated homogenates is believed to be due to the combined action of proteases and desoxyribonuclease.

The observations presented, together with the results of previous studies, are taken to indicate that the envelope of the isolated liver cell nucleus is a porous structure which allows free passage of a number of high molecular weight substances. The results do not support the theory that the nuclear envelope is composed solely of the chromosomal sheaths of swollen or hydrated chromosomes.

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CYTOLOGICAL CONDITIONS IN ROOT TIP MERISTEM AFTER GROSS ANTAGONISM OF PHENYLMERCURIC POISONING

EILEEN W. E. MACFARLANE

Institutum Divi Thomae, Cincinnati, Ohio and Palm Beach, Florida, U.S.A.

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An analysis has been made of the dividing cells in the root tips of the onion *Allium cepa* L. after different degrees of successful gross antagonism of fatal phenylmercuric poisoning by cellular extracts and thiol compounds. Judging by the comparative gross reactions in the enlarging cells the antagonists apparently reduced the effective concentrations of basic phenylmercuric nitrate (PMN) and of phenylmercuric hydroxide (PMOH) 50 per cent to 95 per cent (9). It was of interest to see whether the dividing cells were also protected, particularly because the organic mercurials produce radiomimetic effects (6) and may also be mutagenic (7).

The three ascending degrees of spindle disturbances (13) are 1) changed orientation; 2) partial inactivation of spindle synchronization; and 3) complete inactivation of spindle or c-mitosis (stathmokinesis). Both the last two reactions give rise to polyploid nuclei and are produced by colchicine and the mercurials. We have included the first two stages in our Grade 2 and c-mitosis in Grade 3 of cytological reactions. Cytological effects of the mercurials that have not been reported with colchicine are: chromosome erosion, decreased condensation at metaphase, chromatin bridges at anaphase, a low frequency of chromosome fragmentation, chromosome stickiness, and a disturbed nucleolar cycle (6). These are radiomimetic effects, and the mercurials also induced sectorial somatic mutations and sterility in plant shoots (9).

METHODS

Bulbs of *Allium cepa* L. of the yellow globe type were germinated in the dark on glass jars full of tap water. Treatments were given for an hour or continuously for three days on similar jars at 25° ± 2° C. The antagonists used were cysteine hydrochloride, glutathione and an aqueous solution of alcoholic extract of yeast. The local tap water was the diluent for these antagonists and for the mercurials (9). Tips were fixed in acetic-alcohol [1:3], stained in aceto-carmine and mounted as permanent squash preparations, with or without counterstaining with fast green.

Experiments were run in Palm Beach, Florida and at Cincinnati, Ohio.

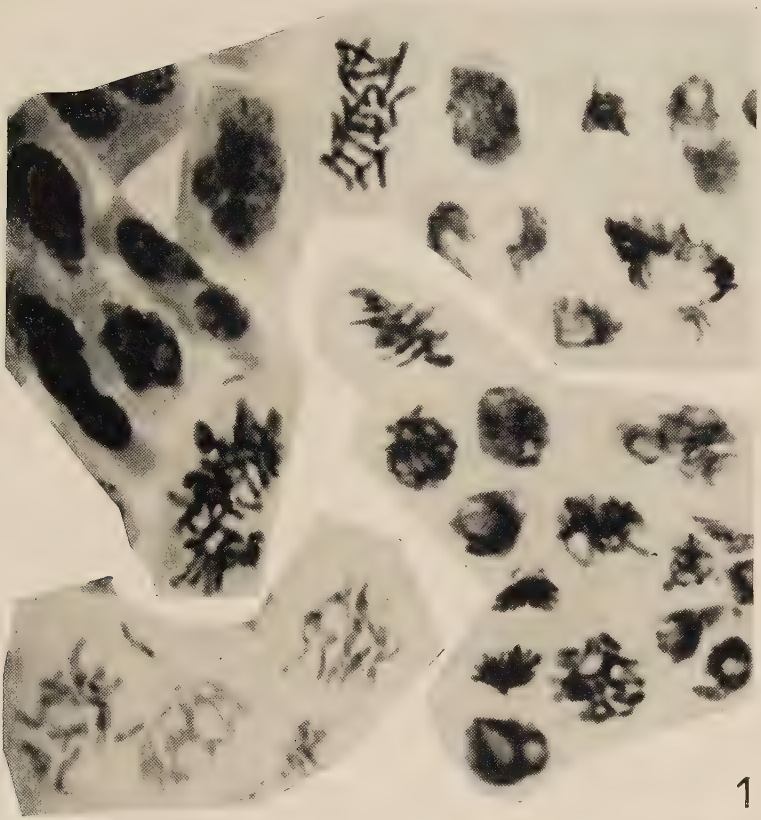
Reactions classified. To facilitate the use of the onion root as an indicator for toxicity and for tabulation, the reactions have been classified into four grades. Gross reactions originating in the youngest enlarging tissue behind the meristem have been designated as Grades I–IV. The sub-terminal swellings produced are named c-tumors because this is a colchicine-like reaction. The cytological reactions of the terminal dividing cells have been designated as Grades 1–4 (6 -8). The three lower gross and cytological reactions in each grade are produced by similar dilutions of the poisons, with a time lag, as well as greater recuperative power (reversible reactions), in the dividing cells. Essentially, the reactions and the causal concentrations of PMOH in Cincinnati water for an hour are:

Gross	Cytological
Grade I – Slight torsion; sometimes accelerated growth rate. Below 2.0×10^{-6}M.	1. Increased frequency of prophase and telophase figures; some chromosome fragments and chromatin bridges at anaphase in 24 hours.
Grade II – C-tumors followed by recovery of normal growth. In $2–5.2 \times 10^{-6}$ M. Alone or mixed with Grade III.	2. Spindles inefficient, may be tilted, multiple, or multipolar; mitotic timing upset; some 4n restitution nuclei and polynucleate cells; sticky chromatids; fragments; extra nucleoli and nucleoli persisting on spindle.
Grade III – C-tumors with slow growth recovered in clubbed tips. Often mixed with II or IV. In $3.4 - 8 \times 10^{-6}$ M.	3. Spindle formation inhibited; full c-mitosis with blocked metaphases followed by amitotic phase in 2n and 4n nuclei.
Grade IV – Death, first in older elongating cells. Minimum LD $100 = 8 \times 10^{-6}$ M.	4. Clumping of mitotic chromosomes; destruction of nucleoli and pyknosis in resting nuclei. Begins at 3.4×10^{-5} M given for an hour.

Fig. 1. Root meristem one day after PMOH 10 ppm and 0.01 per cent cysteine for an hour. Recovery of c-mitosis and early spindle-formation (reactions 3 and 2) after amitotic phase. *Upper left:* polynucleate and polyploid cells. *Upper right:* tri-polar spindle and chromosome fragments. *Upper centre:* metaphase arrested with chromosomes on equator (cysteine effect). *Lower right:* Lobed nuclei, enlarged nucleoli. *Lower left:* c-mitosis type of arrested metaphase with scattered diplochromosomes (mercurial effect). *Scale* ×1100.

Fig. 2 a (left side). Root meristem 5 hours after PMOH 10 ppm for an hour. Telophase with tilted (diagonal) spindle. Resting cells with 3 or more nucleoli. All cells entering amitotic phase prior to pyknosis and death. *Scale* ×1200.

Fig. 2 b (right side). Root meristem one day after PMOH 10 ppm and 0.01 per cent cysteine for an hour. Recovery of mitosis after amitotic phase. Telophase with chromatin bridges; polynucleate resting cells. *Scale* ×1200.



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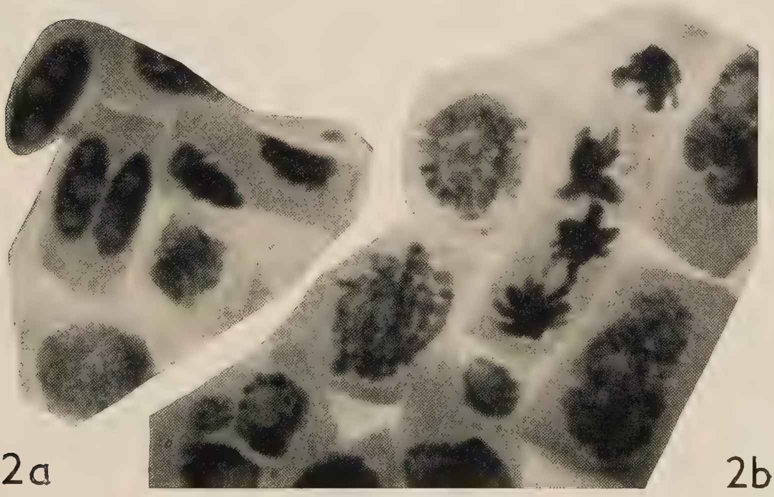

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2b

TABLE I

Cytological reactions in *Allium* roots after an hour's exposure to mercurials and antagonists.

Poison in ppm	Antagonist Per cent	Time Posttreatment Hours	2n Divisions	Extra nucleoli	Polyploid Divisions	c-mitosis	Final Grades Reaction	Abnormalities a Remarks
PMN 10	—	0	–	±	–	+ +	3, IV	2, 4, 5, 7
PMN 10	AYE 1	0	+ +	–	–	–	1	1, 6
,, ,,	,,	5	–	±	–	±	3	2, 5
,, ,,	,,	10	+ +	±	±	±	3–2	5
,, ,,	,,	24	+	+	±	±	3–2, III	9, 10, equiv. PMN 2
,, ,,	GSH 0.5	0	+ T	–	–	±	3	5
,, ,,	,,	2	+ T	–	–	±	3	5, 2n and 4n c-mi
,, ,,	,,	5	–	–	–	–	2	Amitotic phase
,, ,,	,,	24	+	–	–	–	1, II–III	Recovery
CT ,, ,,	GSH 0.001	24	+	+	±	–	2·1 III	2, 5, 10
PMOH 10	—	0	±	±	–	±	3–4	4, 5, 8, 2n & 4n, c-mi
,, ,,	—	5	–	+	–	+	4, IV	1, 3, 7, 8
,, ,,	AYE 1	0	+ +	–	–	±	2 (3)	1, 6, 9
,, ,,	,,	5	–	±	–	±	3	2, 3
,, ,,	,,	24	+	±	–	–	2–1, III	9, recovery
,, ,,	GSH 0.5	0	+	–	–	±	1-3	2, 4
,, ,,	,,	5	±	–	–	±	1–3	5, 10
,, ,,	,,	24	+	–	–	–	1, III	Meristem recovery
CT ,, ,,	CSH 0.01	0	+ +	–	±	–	1-2	1, 2, 5, 6, 10 Excess
,, ,, ,,	,,	5	–	+ +	–	+	3	2, 3, 7, 9
,, ,, ,,	,,	10	–	–	–	–	2	Amitotic
,, ,, ,,	,,	24	±	+ +	±	+ + +	3–2, III	1, 2, 3, 5, 6, 7, 9, 10, equiv. PMOH 2
CT none	AYE 1	24	+ +	+	+ +	+	3–2, I–II	9 bi-and micronuc recovery
CT ,,	GSH 0.5	0	+ + +	–	±	–	1–2	2, 5, 6, stimulation
CT ,,	,,	5	±	±	±	–	1–2	2, 5, 6, 7, enlarging injured
CT ,,	,,	24	+ +	+	+	–	1, II	2, 5, 6, 7, enlarging injured
CT ,,	GSH 0.01	0	+ +	±	±	–	1	2, 5, 6
CT ,,	,,	24	+ +	+	+	–	1, I–II	5, 9 (bi-nucl.)
CT ,,	CSH 0.01	0	+ + +	±	±	–	1–2, I	5, 6, 9, stimulation
PMN 10	CSH 1	0	+	–	±	–	2, IV	1, 2, 5

± Rare, 1–3 per tip, + Few, 2 in 2–3 high power fields
+ + Normal or 2-5 each high power field, + + + many.
T – telophase, CT – Cincinnati tap water, all others Palm Beach water.
Abnormalities. 1-tilted spindle, 2 – prophase arrest, 3 – multipolar spindle, 4 – prominent heterochromatic knots, 5 – chromatin bridges, 6 – turgid nuclei, 7 – diplochromosomes, 8 – clumped mitosis, 9 – polynuclei, 10 – chromosome fragments, 11 – ring fragment.
AYE – Alcoholic yeast extract, CSH – cysteine, GSH – glutathione.

The effects on spindle formation below grade 4 are reversible. Subsequent to these primary reactions the dividing cells go into an amitotic phase of varying duration and then pass into reactions 3, 2, and 1 in recovering to normal, or directly into reaction 2 in recovery phase. A precocious ageing often follows with differentiation and reduction of the volume of the meristem.

EVALUATION OF ANTAGONISM BY CYTOLOGICAL REACTIONS

Short treatments. The lowest concentration of a mitotic poison which causes chromosomes of dividing cells to clump has been called the effective mitotic concentration or EMC by O'Connor (12). In both the tap waters, the concentration for mitotic chromosome clumping for an hour's exposure was PMOH 10 ppm (3.4×10^{-5} M), which was ten times the MLD for the elongating tissue (9). This was the concentration used in these antagonism experiments and it produced a grade 3–4 cytological reaction in an hour by itself (Table I). Occasionally, a nucleus was arrested in telophase during treatment and for a few hours after (Fig. 2 a). PMN at 10 ppm was one half the molar strength of the PMOH 10 ppm and therefore did not clump chromosomes. It gave a grade 3 reaction during an hour with some accumulation of blocked metaphases. Table I gives a summary of the reactions in the dividing cells that occurred with successful gross antagonism after short treatment. It should be remembered that mitotic poisoning below Grade 4 was a reversible reaction.

Temporary protection with no obvious cytological effects at the end of the hour of treatment was obtained only with PMN 10 ppm and yeast 1 per cent[1] and PMOH 10 ppm and cysteine 0.01 per cent. Five hours later, spindle formation was inhibited in both sets of roots and some c-mitosis appeared (grade 3). Later, some cells regained spindles in grade 2 of recovery, rapidly after the yeast and slowly after cysteine antagonism. One day after the latter there was a marked accumulation of blocked metaphases (after an earlier amitotic phase) as well as some irregular grade 2 spindle mitosis. The presence of multinucleate and polyploid resting cells was indicative of an earlier primary grade 2 reaction as shown in Figs. 1 and 2 b. Although the cysteine was in excess 23.5 times, the cytological reactions were for the most part similar to those after an hour's exposure to PMOH 2 ppm (6), or to 0.25 ppm continuously for twenty-four hours. This is equivalent to an antagonism efficiency of 80 per cent while that for the enlarging cells in the same treatment was 85 per cent (8) (Table I). It is suggested that the effective internal concentration of the poison that penetrated the meristem was not over 0.25 ppm (8.5×10^{-7} M).

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[1] Roots immersed in the yeast extract did not grow at all and developed c-tumors upon return to water. There was a rapid differentiation of the primary meristem and then lateral roots were produced.

Glutathione at 0.001 per cent (3.2×10^{-5} M), although only two times in excess, gave good protection against spindle arrest by PMN 10 ppm (1.6×10^{-5} M). However, twenty-four hours after exposure, radiomimetic effects were evident as chromatin bridges and fragments (Table I). Grossly normal growth did not occur after this treatment. The cytological effect was a little less than that caused by an hour in PMN 2 ppm (or by a day in PMN 0.05 ppm), indicating again an 80 per cent antagonism efficiency and an effective internal concentration of the poison of scarcely 3.8×10^{-7} M. Here too, a few dividing cells had the chromosomes arrested on the equator as described below.

Glutathione at 0.5 per cent, and several hundred times in excess[1], protected the meristem almost completely against inhibition of spindle formation by 10 ppm of both PMN and PMOH, but caused an amitotic phase of short duration a few hours later. A day later mitosis was proceeding at below normal rate. The reaction was similar to that caused by PMOH 0.5 ppm indicating a 95 per cent efficiency of antagonism which is similar to that given the enlarging cells. Here again, chromosome damage was evident a day after exposure as chromatin bridges and fragments (Table I, abnormalities 5 and 10). Similar effects were recorded after an hour in PMOH 0.5 ppm and after twenty-four hours in PMOH 0.1 ppm in Cincinnati water (6) (Table II).

Usually, the thiol-mercury solutions reduced mitotic rate for some hours after treatment. When some dividing cells went into c-mitosis (no spindle) as with the poison alone, a few showed an uncommon type of metaphase arrest with the chromosomes on the equator and irregularly condensed. Some of these blocked divisions failed to proceed to anaphase and formed tetraploid recombination nuclei from the metaphase position. An amitotic phase followed.[2]

This equatorial type of metaphase arrest was also observed after short exposure to glutathione 0.001, 0.01, and 0.5 per cent alone, as well as after cysteine 0.01 per cent alone. The glutathione caused an initial rise in mitotic rate and more polyploid divisions twenty-four hours afterwards than the cysteine.[1] In these cases of anaphase failure it has not been determined whether the spindle which had presumably formed, became functionless, or if it dissolved as with colchicine (14). The pairs of sister chromatids did not diverge to give X-shaped c-pairs, instead, while still on the equator they

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[1] The thiols were first used in large excess in strengths employed by our colleagues in artificial enzyme systems. Exposure for an hour to cysteine 0.125 per cent and stronger in combination with the mercurials (or alone) caused death of the root tissue behind the meristem more rapidly than with the poison alone and usually during treatment. But cytologically the meristem showed active mitosis in presence of mercury and thiol and an apparent protection against the poison. Tips fixed a day after treatment (the roots were water-logged and flaccid) showed loss of nucleoli in some larger nuclei and an occasional bridge at telophase and an occasional clumped metaphase, indicating a gradual onset of lethal changes before the meristem was "fixed" by the lethal mixture. PMN 10 ppm and cysteine 1 per cent for an hour acted as a fairly good cytological fixative.

[2] Both thiols alone in non-lethal concentrations tended to produce an amitotic phase a few hours after short exposure with arrest in interphase and early prophase. At the same time both the nucleus and the protoplast as a whole became somewhat hypertrophied.

TABLE II

Cytological reactions in *Allium* roots with antagonism of lethal concentrations of PMN and in antagosists alone in prolonged exposure. Cincinnati tap water.

MN m	Antag-onist per cent	Length of treatment Hours	2n Divi-sions	Extra Nucleoli	Poly-ploid Divi-sions	c-mi-tosis	Cyto-logical Grade	Gross[2] Grade	Abnormalities and Remarks
.2	—	24	–	+ +	–	+ + +	3	III, IV	9
,	AYE 0.25	72 (a)	–	+ +	–	+ +	3	II, IV	9, no spindles
		(b)	+ +	+ +	+	+	3 -2		Recovery, 3, 5, 7, 9
,	CSH 0.01	72	+ +	+ +	+ +	–	2	I	5, 9 cell plate inhibition
,	GSH 0.01	72	+ +	+	±	±	3–2	I	(No c-tumors)
.5	—	24	–	+	–	–	4	IV	Necrosis
.5	AYE 0.25	24	–	±	–	+ +	2,3		3, 4, 5
		[3]48/72 H_2O	+ +	+ +	+ +	+	3 2	II, III	5, 7, 9, 10 Divn.-stimulated same as AYE alone (with more polyploidy)
	AYE 0.25	48	–	+	–	+	3		9 Micronuclei
		48/24 H_2O	+	+ +	±	±	3 -2	III	Recovery in H_2O Tiny c-tumors.
	CSH 0.01	72	+	±	+	–	2	II	2, 5, 6 low stainability
	GSH 0.01	72	+	–	±	–	1	I	2, 5, 6 low stainability

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[1] Cysteine alone also produced vacuolization of cytoplasm. With an hour's exposure to the low concentrations (8 and 16 × 10–⁴ M) more pairs of sister nuclei were dividing synchronously at the end than in control tips. There was a tendency for increase in frequency of prophases and metaphases and some chromosomes were negatively heteropycnotic. Examples of the following were seen very infrequently: tetraploid divisions, extra nuclei, anaphase bridges, two spindles in the same cell and eroded chromosomes. The latter were associated with an occasional micronucleus in the one root in which they were found, and these may have arisen from chromosome fragments.

[2] Final gross reaction after return to tap water. Growth in length arrested during treatment.

[3] After return to tap water.

started independently and non-synchronously to uncoil and enter the resting condition, somewhat as described by Wilson (17) in onion root tips treated with antibiotics. Higher concentrations of the thiols alone caused chromosome stickiness in anaphase of nuclei caught in division.

After exposure to the combinations of poison with yeast or glutathione where the antagonist was in great excess, the cytological effects tended to be more characteristic of the antagonist, although not exclusively. However, there was a more rapid recovery after return to tap water, and the cytological reaction was less marked than that produced by either substance alone[1] in the same concentration. This points to an interaction between the mercurial and the antagonist in the medium. In the antagonism of PMOH 10 ppm by cysteine 0.01 per cent (×23.5 in excess), the cytological reaction was mostly characteristic of the PMOH. But the influence of the cysteine was shown in an occasional nucleus with negatively heteropycnotic chromosomes at metaphase as in the centre of Fig. 1. This appears to be a cytological demonstration of the instability of the combination of the mercury with the sulfhydryl group of the thiol.

In general, the cytological reaction (like the gross effects), after antagonism, resembled that of a much reduced concentration of the poison alone.

Continuous exposure. The strongly growth arresting effects of the antagonists limited the usefulness of tests in prolonged exposure. The PMN 0.2 ppm was antagonized for 72 hours by both thiols at 0.01 per cent and the mitotic rate was normal, although no growth in length occurred in the solutions. Normal growth was renewed upon return to tap water (9). When yeast extract 0.24 per cent was used with PMN 0.2 ppm, there was an accumulation of blocked metaphases in c-mitosis in 72 hours in some tips, while others had recovered spindle mitosis at normal rate. The reaction was similar to that found 24 hours after an hour's exposure to PMN 10 ppm and 1 per cent of yeast extract (Table I). PMN 2.5 ppm, which alone was fatal to all roots in a few hours, was antagonized by 0.25 per cent yeast extract for 48 hours, and both normal and c-mitosis were present at the end of the first day. Even after three days in tap water post-treatment, some c-mitosis was still found. Normal division and some c-mitosis were found 24 hours after 48 hours in yeast extract 0.25 per cent alone. The thiols alone given for three days caused a reduction in the mitotic rate, and a few polyploid divisions appeared. Chromatin bridges were found occasionally after continuous exposure to either cysteine or glutathione. Anaphase bridges were present after antagonism in prolonged exposure.

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[1] The cellular extracts arrested mitosis. Yeast extract at one per cent alone in an hour reduced the mitotic rate with increased frequency of prophases. It caused a grade 2 cytological reaction with inefficient spindles and non-synchronized anaphase movements leading to 4n restitution nuclei and multinucleate cells. Two spindles of equal size were seen in each of two cells and another root showed a blocked metaphase (c-mitosis) with very short over-condensed chromosomes. The meristematic tips were plump and strongly Feulgen positive. The yeast extract contained amino- and nucleic acids (11) which may depress mitotic rate.

DISCUSSION

Depression of the enzymes of the respiratory chain by PMOH and PMN can be prevented by yeast extract and by cysteine and glutathione present in excess (2). Gross antagonism of lethal concentration has also been accomplished for onion roots with these antagonists (9). Cysteine 10^{-4} M enhanced the depression of the succinoxidase system by PMN 2.5×10^{-6} M (4), but, in the absence of air, cysteine at 5×10^{-6} M and 1×10^{-4} M could protect the enzyme and antagonize PMN up to a concentration of 4.5×10^{-5} M.

It has been pointed out that in the onion root tip the oldest meristematic and youngest enlarging cells can pass into normal growth by elongation after temporary arrest caused by an hour in concentrations of organic mercurials similar to those that give a 50 per cent depression of the succinoxidase system *in vitro,* which was 10^{-6} M. This type of concentration also caused reversible reduced permeability of root cells, reversible inhibition of spindle formation in dividing cells and irreversible polyploidy and radiomimetic effects on chromosomes (6).

Clumping of chromosomes did not occur until the concentration was similar to that which produces a 50 per cent depression of the succinic dehydrogenase system *in vitro* or 10^{-5} M for an hour (6). O'Connor (12) found that chromosome clumping in embryonic chick tissue *in vitro* first appeared in concentrations of NaF in which depression of aerobic glycolysis also began. Qualitative tests have shown that dehydrogenases are detectable in, and perhaps characteristic of, plant meristems (16). Dividing cells were protected by extract of yeast, by cysteine and glutathione in excess, against concentrations of the mercurials which alone would cause clumping in mitosis. Even spindle arrest was prevented in some experiments, but radiomimetic effects were not eliminated. These are produced by very high dilutions of the organic mercurials. Intracellular traces of the heavy metal poison probably result from the unstable combination of the —SH reacting mercurials and the antagonist (Fig. 1). The elimination of gross and histological effects by antagonism of the mercurial with thiols or yeast extract may not always protect the chromatin from the radiomimetic effects of the poison. It is known that concentrations below the c-mitotic strength, which do not inhibit spindle-formation, are more effective in producing radiomimetic effects on chromosomes (6). The thiols alone caused chromosome stickiness in nuclei caught in division but very rarely anaphase bridges nor fragments in those entering mitosis hours after treatment.

The poison may reach the meristem chiefly from older absorbing region above (8). The concentration in the former estimated by the cytological reaction twenty-four hours after exposure to poison and antagonist was similar to that produced in the elongating cells by an hour's exposure to the same mixture. Non-irritant doses of phenylmercuric acetate applied to the vaginal mucosa of the rat accumulated in the liver and kidneys (5). Radiomimetic effects of mercurials have not been reported in higher animals but leucocytopenia was reported in man after use of mercurial diuretics (15). The onion root tip appears to react to some mitotic poisons and inhibitors in a manner similar to animal cells (3, 10).

For dividing cells there is no minimum dose of x-rays that will not cause chromosome damage, and the same may be true of mercurials and perhaps of other —SH inhibitors. Mutations and chromosome breaks have been ascribed by Barron (1) to the effects of substances with "mild oxidizing powers or thiol combining properties" which cause molecular aggregation by —S—S— linkage in the chromosomes.

Considerable excess of yeast extract was needed to give protection to the root tissues (9). Although recent tests made in these laboratories for sulphur in the extract have been positive, the amount is apparently small. The fact that the cellular extract, and also naturally occurring sulfhydryl compounds, protected growing cells in a large measure from mercurial poisoning suggests the possibility of an auto-protection mechanism against enzyme poisoning in living tissues perhaps originating in dying and dead cells. This could be related to the wound hormones and wound-healing promotion by cellular extracts as reported by Nutini (11). Antagonism of gross tissue damage may reduce the physiological concentration of an enzymic poison to one that does not effect cytoplasmic function immediately and yet degrades the euchromatin and genes.

SUMMARY

1. The dividing cells of onion root tips were protected by yeast extract, glutathione and cysteine in excess against PMOH 3.4×10^{-5} M which alone caused clumping of chromosomes in an hour's exposure. Spindle inhibition

was not eliminated, but in concentrations of PMOH and of PMN near 10^{-5} M it was delayed or more rapidly overcome. The effect was similar to an 80 to 95 per cent reduction in the concentration of the mercurials.

2. A day after antagonism of PMOH 3.4×10^{-5} M with cysteine 8×10^{-4} M for an hour, a majority of dividing cells showed mitotic abnormalities characteristic of narcotic concentrations of PMOH. A few showed arrest of metaphase chromosomes on the equator as produced by cysteine alone.

3. Successful antagonism of lethal concentrations of PMN in prolonged exposure was accompanied by accumulation of blocked metaphases. Spindle formation was renewed after return to tap water.

4. In all experiments, even when gross effects were practically eliminated by antagonism with thiols, radiomimetic effects (chromosome fragments and chromatin bridges) appeared in dividing cells after exposure.

5. Effective concentrations of all antagonists by themselves caused mitotic and chromosomal abnormalities.

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ELECTRON MICROGRAPHS OF MITOTIC CELLS OF THE EHRLICH MOUSE ASCITES TUMOR IN THIN SECTIONS

CECILY CANNAN SELBY[1]

Sloan-Kettering Institute for Cancer Research, New York, N.Y., U.S.A.

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Despite its great contributions to other fields of cytology, electron microscopy has, so far, contributed little to our understanding of the morphology of nuclear and mitotic structures. This is primarily because the nuclear region does not spread thin enough for direct observation in tissue culture or in smear preparations. Whole nuclei have, therefore, been studied successfully only in tissue sections. The use of ultra-thin sectioning techniques introduces the serious problem of recognizing structures familiar to the cytologist in such thin sections, and of reconstructing a three-dimensional picture of these from a random selection of 50 to 150 sections through a cell. Mitotic figures have been particularly difficult to study because fixatives, such as alcohol, acetic acid and Flemmings solution, most favorable for delineation of these structures under the light microscope, produce such coarse cyto-

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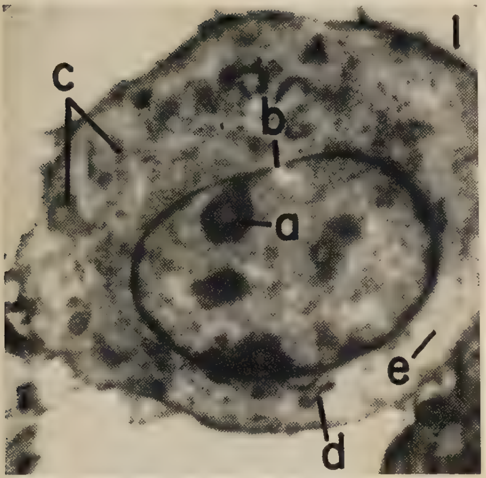
Fig. 1. Resting cell of Ehrlich mouse ascites tumor. See text for description. 5400 ×.
Fig. 2. Early prophase. The chromosomes are taking form near the nuclear membrane. 4800 ×.
Fig. 3. Early prophase. The chromosomes are lined up along the nuclear membrane. 4800 ×.
Figs. 4 and 5. Prometaphase. Partial dissolution of the nuclear membrane is illustrated. 4200 ×. 3800 ×.
Fig. 6. Metaphase chromosomes showing periodicity. 4200 ×.
Fig. 7. Metaphase. The chromosomes have divided longitudinally. 4800 ×.
Figs. 8 and 9. Metaphase. 4200 ×.
Fig. 10. Metaphase. In the upper center of the cell, the elongated fiber may be a chromosomal fiber of the spindle or a thinly sectioned portion of a chromosome. 3800 ×.
Figs. 11 and 12. Metaphase. The chromosome halves have separated. 4800 ×. 4200 ×.
Fig. 13. Metaphase. Note the periodicity of the chromosomal contours. 4200 ×.
Fig. 14. Anaphase. The ballooning of the mitochrondria and the presence of large fat drops usually indicate ageing of the cell. 5000 ×.
Figs. 15 and 16. Anaphase. The chromosomal fibers of the spindle are clearly shown. 4200 ×. 3800 ×.
Fig. 17. Telophase. The cytoplasmic bridge is constricting. There are no indications of interzonal fibers of the spindle. 5400 ×.
Fig. 18. Daughter cells. The nuclei have reformed. 3300 ×.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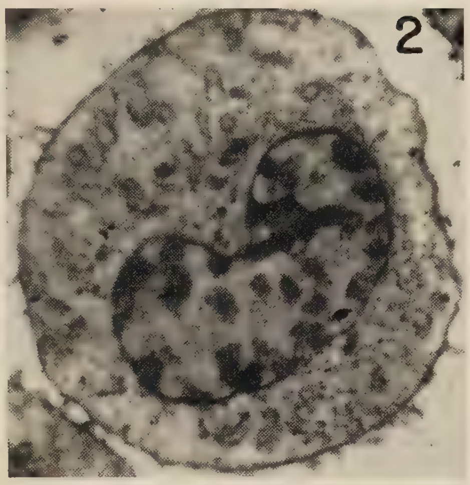
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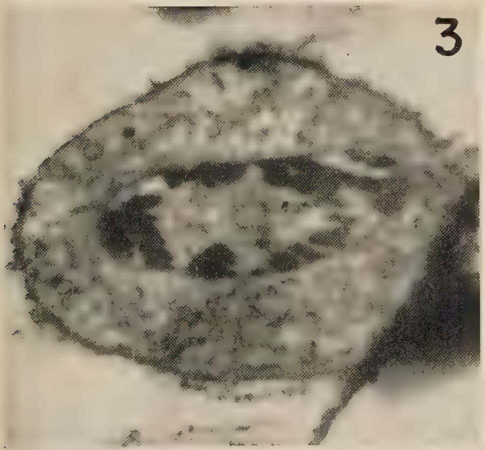
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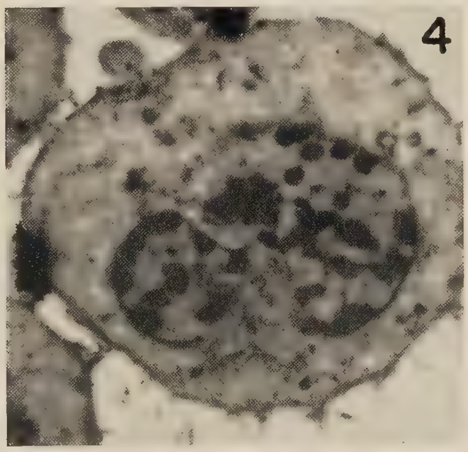
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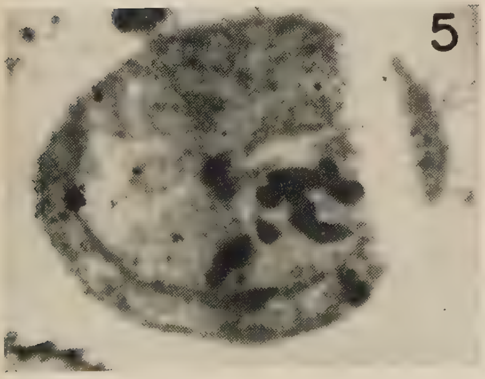
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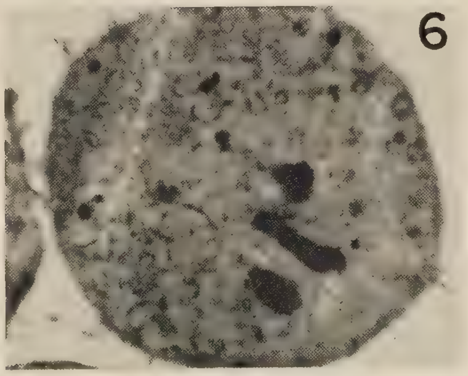
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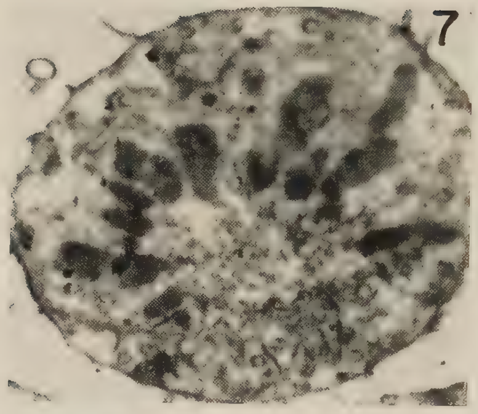
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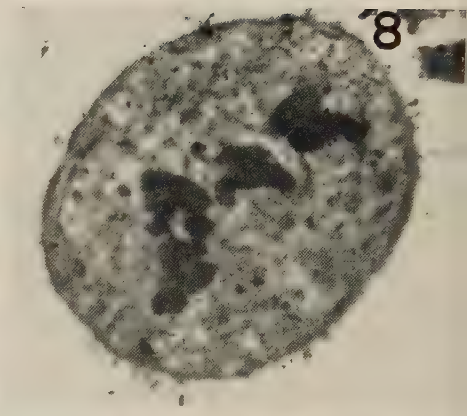
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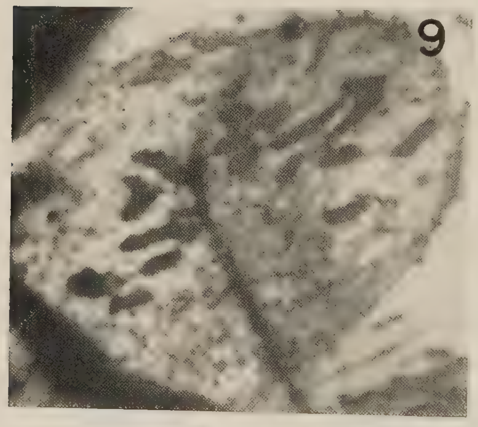
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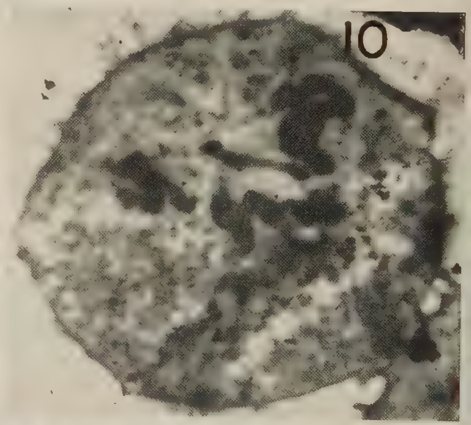
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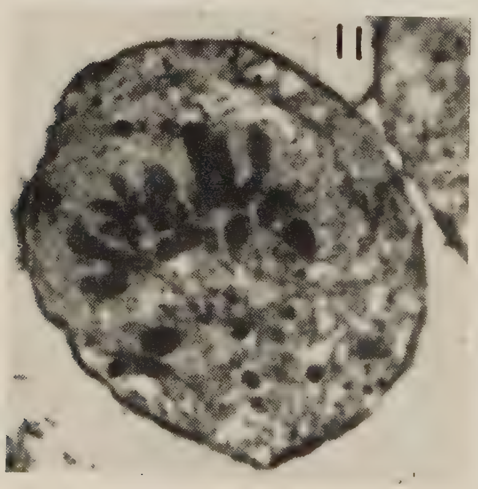
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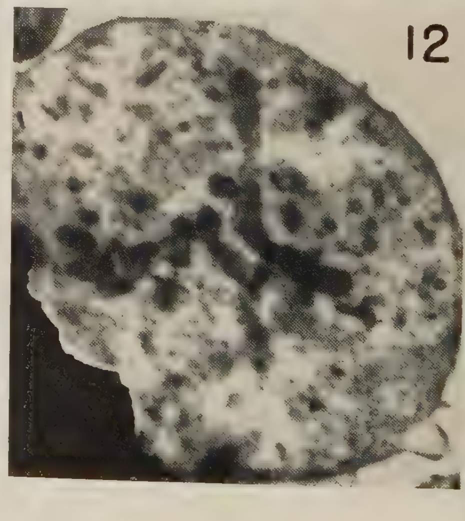
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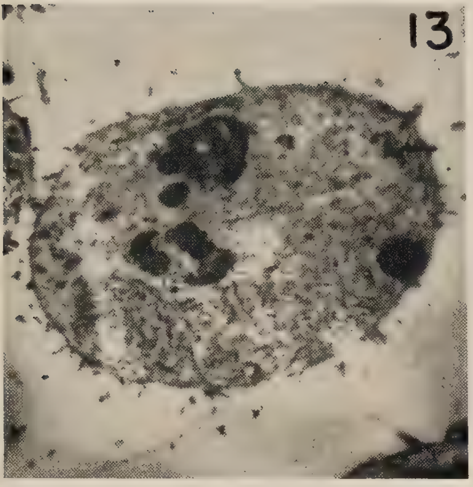

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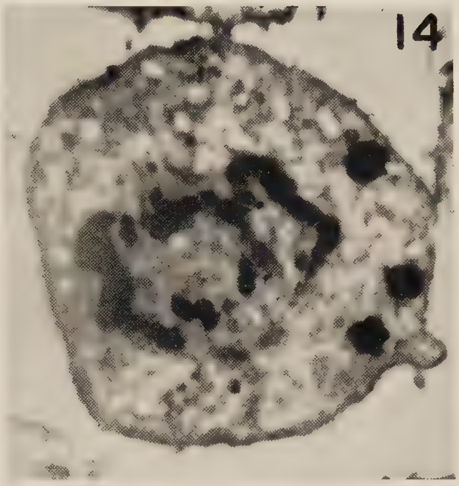

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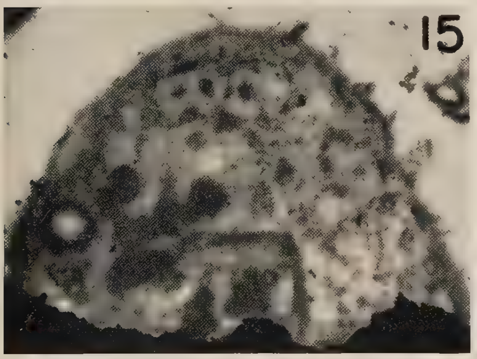

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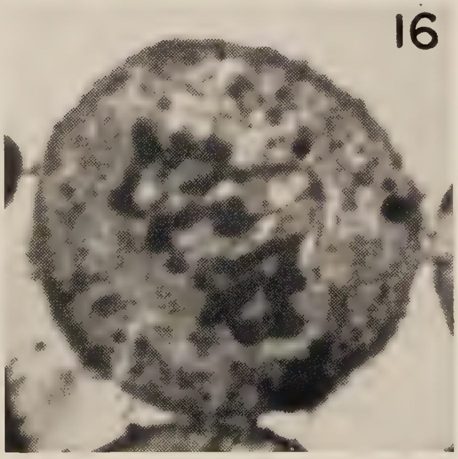

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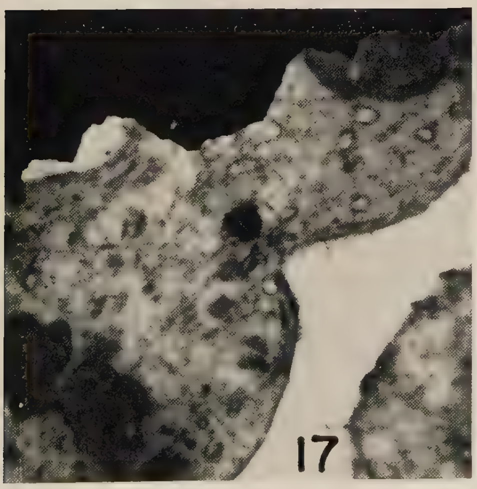

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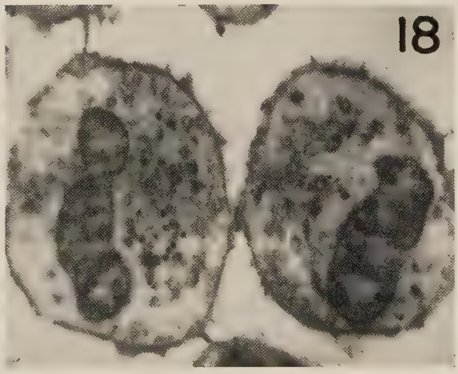

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plasmic coagulation as to render the cell unrecognizable under the higher magnifying power of the electron microscope. In particular, it is difficult to distinguish fixation-induced fibrillation or granulation from such structures which might be present in the living cell (2, 3). In neutral formalin or osmium-fixed cells of *Allium* the spindle-fibers evident in acid-fixed material have not been observed (7, 16, 17). Rozsa notes only a preferred orientation of the cytoplasmic ground substance in the region of the spindle and equatorial disk and concludes, therefore, that the spindle in acid-fixed preparations must result from partial rupture of the cytoplasmic gel which is simply under tension.

We have found the Ehrlich mouse ascites tumor propagated in the peritoneal cavity of the mouse particularly favorable for morphological studies because the most rapid and thorough penetration of osmic acid is facilitated by introducing it directly into the ascitic fluid surrounding the cells. These cells are also convenient for study of the mitotic apparatus since the malignant cells multiply so rapidly in the peritoneal fluid that, from a few to ten days following implantation, a high percentage of mitotic figures may be found. During the course of work developing ultrathin sectioning methods a series of thin sections of such mitotic figures were obtained. These are being presented here, although they do not represent the very finest resolution available at the present time, because of the new information they do contain and because the subject is not being pursued further by the author.

Immediately after removal from the animal, the ascites cells were centrifuged free of ascitic fluid and resuspended in a solution of 1 per cent osmic acid buffered in Locke-Ringer's solution. The final pH of this solution is 7.2 but results with this did not differ from those obtained more recently with Palade's fixative (14) buffered at pH 7.4. After 45 minutes, the fixative was centrifuged off and the cells washed five times with distilled water over a total period of one hour. Dehydration was accomplished by repeated suspension in ethanol and centrifugation; the final pellet from absolute ethanol was embedded in methacrylate (13, 14). Sections were cut with a hand-sharpened steel knife (11) in a modified motor-driven Spencer microtome (12). Embedding material was never removed prior to observation.

Fig. 1 illustrates a typical interphase cell with the enlarged oval or kidney-shaped nucleus and diffuse chromatin typical of the Ehrlich ascites tumor. Fine, and usually constant-diameter, pseudopods extend from the free cell surface similar to those noted at the free surfaces of other cell types (1, 8). The nuclear membrane (b) occasionally appears double (10) while the nucleus (a) has not yet been observed organized into the "coiled" nucleoli common in other cell types (5, 6) and in another mouse tumor (18). Instead, areas of reduced electron density are usually observed in the largest chromatin masses.[1] Mitochondria (c) always exhibit a limiting membrane from which double-walled membranes or septa extend either partially or completely across the mitochondrion (20). Double-walled structures (d) with a total diameter of about 500 Å are sparsely distributed throughout the cytoplasm. By virtue of their size and morphology these appear identical to the sections of membranes (20) or canaliculi (14) described by other authors and presumed to be the locus of the basophilic substance of the cytoplasm (9, 4). Small vesicles (e) with diameters about 700 Å are evenly distributed through the cytoplasm and suggest the endoplasmic reticulum of cultured cells (15, 19). Fig. 2 illustrates early prophase with the chromatin organized into chromosomes which in a later stage (Fig. 3) are aggregated around the nuclear membrane. An early stage of dissolution of the nuclear membrane is evident in Fig. 4 where mitochondria are in the nuclear region in close association with the chromosomes. The nuclear membrane is further dissolved in Fig. 5 while the nuclear and cytoplasmic regions still appear differentiated. Complete dissolution is illustrated in Fig. 6 with cytoplasmic components distributed uniformly through the cell.

Figs. 7–12 illustrate various views of metaphase. Chromosome division is evident in the polar view of Fig. 7 and in Fig. 8. Figs. 11 and 12 show the metaphase plate with separation of the daughter chromosomes. Movement of these toward the poles is illustrated in Figs. 14, 15, and 16. The chromosomal fibers, as seen in Figs. 15 and 16, are the only elements of the spindle which have been observed as osmiophilic fibers. In the interzonal region there are no fibers, but only a general orientation of cytoplasm. The location of an osmiophilic particle at the apex of the spindle in Fig. 15 is suggestive of its identity with the centriole, but the possibility of coincidence with another cytoplasmic particle at this position is not excluded. In telophase (Fig. 17) orientation of the mitochondria and cytoplasmic vesicles in the interzonal region perpendicular to the plane of division is particularly clear. Reconstruction of the daughter nuclei is illustrated in the last figure where orientation of the chromatin within the reconstructed membrane is clear, together with a localization of granules in the central region.

One interesting fact which arises from these observations is that the chromosomal fibers are the only component of the spindle to fix as a fibrous structure in neutral osmic fixation. Although the possibility is not excluded that there are other osmiophilic fine fibrous components that have not yet been sectioned in an angle favorable for recognition this is extremely unlikely in view of the large number of mitotic figures which have been observed. This result suggests that birefringence and other properties associated with the spindle in the astral and interzonal regions are a result of orientation of cytoplasmic particulates and/or gel, while the chromosomal fibres alone are actual fibers differentiated from the rest of the protoplasm.

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[1] Since the preparation of this manuscript typical "coiled" nucleoli have been recognized in many cells and often in conjunction with the vesicular chromatin mass described.

A large period of between .37 and .40 micra has consistently appeared as either a periodic contouring or variation in osmiophilia in metaphase chromosomes. The failure to observe any internal structure inside the chromosomes may be due to the fact that whatever structure there is in osmic acid fixed material is too fine for resolution in sections of this thickness. The filamentous structure revealed by others after removal of the embedding (17) is suggested only in Figs. 11 and 14 but the introduction of artefact by embedding removal must be kept in mind. Another point of interest arising from this work concerns the constancy in morphology of other cytoplasmic particles throughout division. Swelling of the mitochondria, as in Figs. 11, 14, 17, and 18 is not the general rule and may be related to the age of the cell, as evidenced by the fatty inclusions. Further study will be necessary to elucidate the actual fine structure of the chromosomal fibers and the chromosomes, but this study serves to indicate some initial contributions of thin sections of osmic-acid fixed material to this field of cytology.

SUMMARY

Electron micrographs of thin sections of mitotic figures of the Ehrlich mouse ascites tumor cells fixed in osmic acid buffered at pH 7.2 have revealed the following facts:

1. Chromosomal fibers in anaphase are the only spindle structures fixed with osmic acid at this pH. Other regions of the spindle show only orientation of cytoplasm particulates.

2) Mitochondria appear intimately associated with the chromosomes in late prophase.

3) A periodicity of .37 to .40 micra has been noted in metaphase chromosomes.

4) Cytoplasmic particles remain constant in morphology throughout division.

The author wishes to acknowledge with thanks the technical assistance of Miss Sarah Lichtenberg in all phases of this work, of Mr. Clifford E. Grey in maintenence of the electron microscope and of Mr. Alfred E. Spitzer in photographic services.

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SOLUBLE ANTIGENS IN SEA URCHIN GAMETES AND DEVELOPMENTAL STAGES

P. PERLMANN

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

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In a previous paper (7) results have been reported from a series of serological experiments on the eggs and developmental stages of the sea urchin *Paracentrotus lividus*. By means of the ordinary serological precipitation and absorption techniques, the appearance of at least one saline extractable molecular species of a new specificity, not occurring in unfertilized eggs and early developmental stages, could be demonstrated in 0.14 M saline extracts from 48 hours old plutei. In addition it appeared that egg extracts did not contain any precipitin producing antigens besides those in the extracts from the later stages up to 48 hours development.

A further investigation of the serological properties of the sea urchin gametes and developmental stages has now been undertaken by means of Ouchterlony's precipitation method in a salt-agar gel (4, 5, 6). By this method two or more antigen solutions can be tested at the same time. The identity or non-identity of the antigenic molecules in the different solutions can thus be tested qualitatively and semi-quantitatively.

Rabbit antisera against sea urchin material and antigen solutions were prepared and standardized as previously reported. All antigen solutions were maintained by a phosphate buffer at pH 7.4. All solutions could be stored at —17° C in a refrigerated box for several months without losing activity. As a rule the serological reactions were carried out at 37° C in the same way as described by Ouchterlony (l. c.). Precipitation lines usually appeared on the agar plates after 2 days. After approximately 1 week all lines had appeared and grown strong enough for registration. The experiments were usually stopped after 2 weeks. During the second week no more changes occurred in the number of the precipitation lines. — Serological absorption experiments were carried out in the manner described by Björklund (1, 2). After complete reaction the plates were photographed in a Busch-macrocamera and furthermore, drawings were made by means of a camera lucida.

When 0.14 M saline extracts from jelly deprived and lyophilized eggs of the sea urchin *Paracentrotus lividus* were allowed to react with their homologous antisera, a great number of precipitation lines was obtained. Further

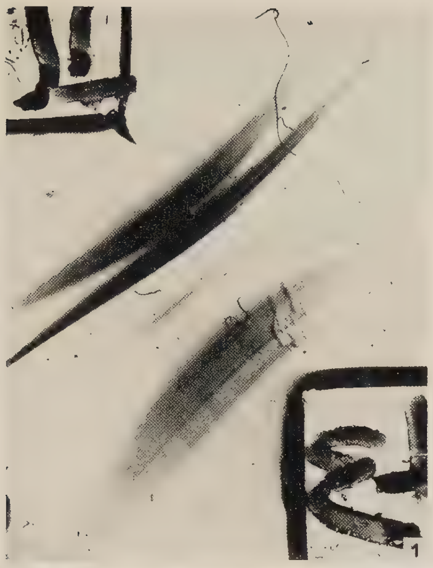


Fig. 1. Egg extract (left), reacting with anti-egg serum (right).

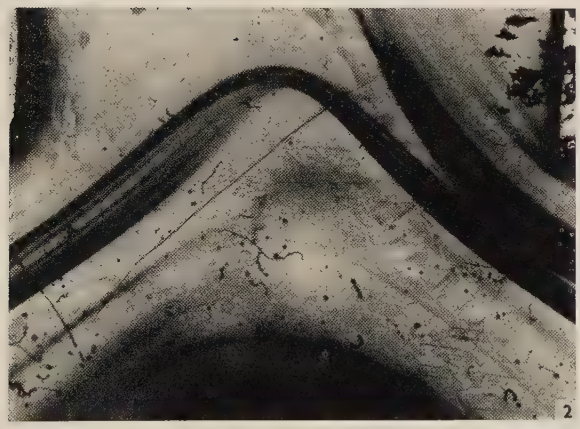


Fig. 2. Egg extract, untreated (right) and after treatment with 40 per cent $AmSO_4$ (left), reacting with anti-egg serum (middle).

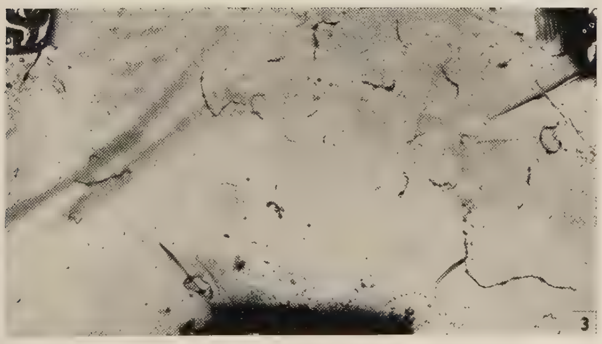
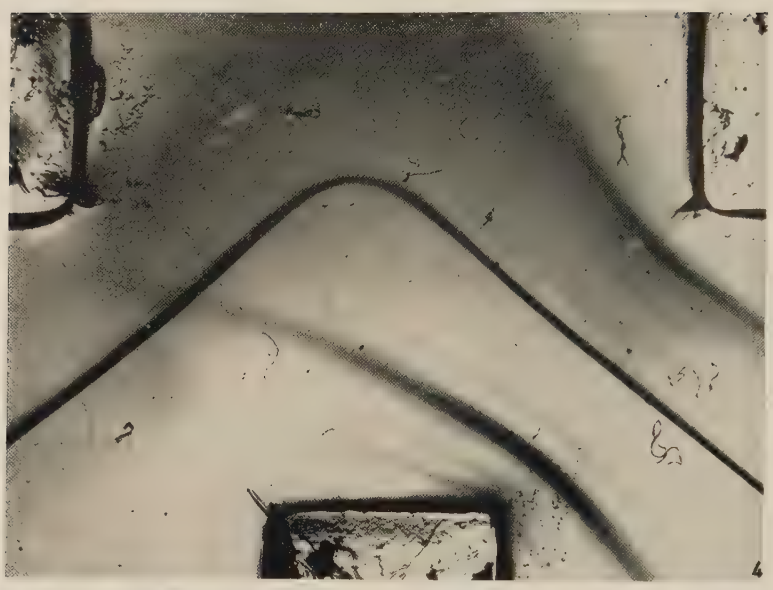


Fig. 3. Pluteus extract (left), reacting with anti-pluteus serum, absorbed with egg extract (middle). No reaction with egg extract (right).



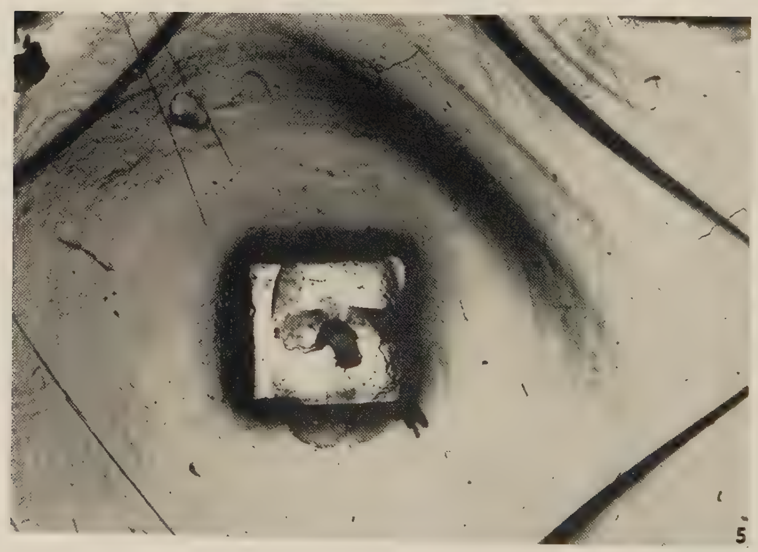


Fig. 4. Egg extract (right) and sperm extract (left), reacting with anti-egg serum (middle).
Fig. 5. Sperm extract (left), egg extract (upper right) and jelly coat solution (lower right), reacting with anti-egg serum (middle).

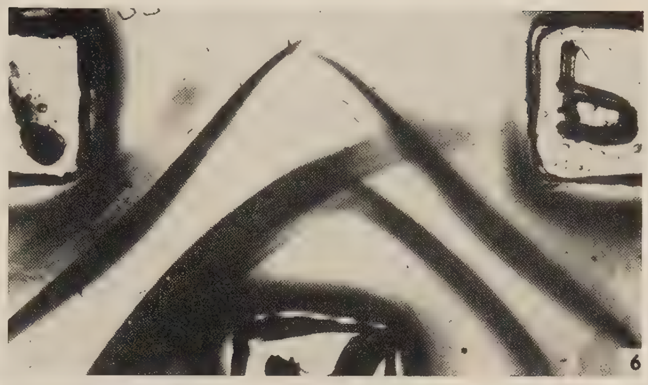


Fig. 6. *Psammechinus* extract (left) and *Paracentrotus* extract (right), reacting with anti-*Psammechinus* serum (middle).

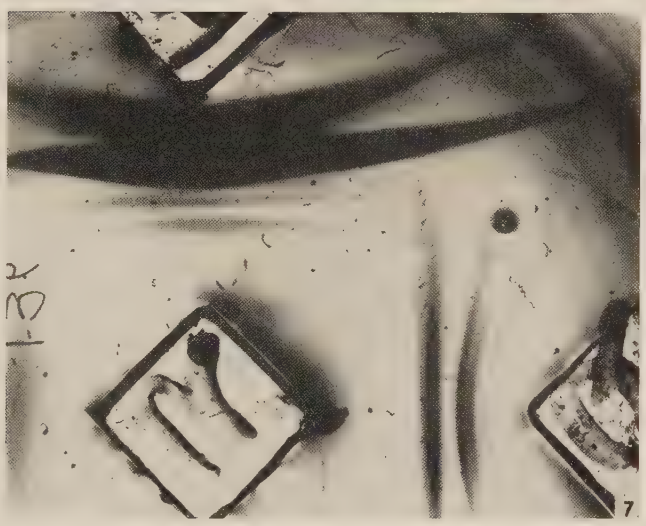


Fig. 7. *Arbacia* extract (upper) and *Paracentrotus* extract (right), reacting with anti-*Arbacia* serum (middle).

experiments showed that at least 10 of those lines really represented molecules with different chemical and serological properties. As can be seen from diagram 1 and from Fig. 1, most of the lines were arranged in at least four main fractions, indicated by the numbers I–IV.

By ultracentrifugation at 65000 × g for 2 hours, some of the components in fraction I could be brought out of solution. Furthermore, in the top layer formed at this centrifugation (3), chiefly components from fraction III and IV could be found. — Dialysis overnight against diluted HCl (pH 3.5) made all antigens insoluble, except most of those in fraction IV. — A further fractionation of the antigens could be achieved by precipitation with $AmSO_4$ in the cold. The experiments were performed as previously described by

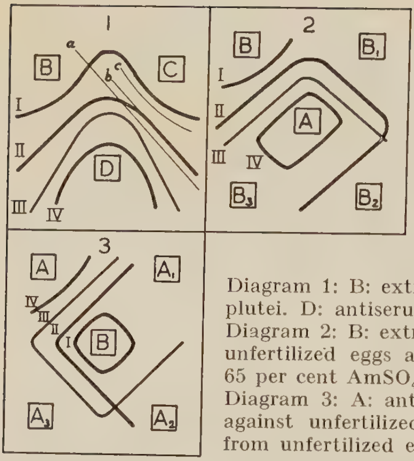


Diagram 1: B: extract from unfertilized eggs. C: extract from 48 hours plutei. D: antiserum against 48 hours plutei.
Diagram 2: B: extract from unfertilized eggs. B_1, B_2, B_3: extracts from unfertilized eggs after precipitation with 40 per cent, 55 per cent and 65 per cent $AmSO_4$. A: antiserum against unfertilized eggs.
Diagram 3: A: antiserum against unfertilized eggs. A_1, A_2, A_3: antisera against unfertilized eggs, absorbed with B_1, B_2, and B_3. B: extract from unfertilized eggs.

Lindvall (3). As can be seen from diagram 2, precipitation with $AmSO_4$ at 40 per cent saturation brought out all antigens in fraction I (Fig. 2). In the saturation-range from 40–60 per cent all antigens in fraction III disappeared from the solution together with some of those in fraction II. Fraction-II-antigens disappeared almost completely, if the $AmSO_4$-saturation was incerased up to 65 per cent. Most of the antigens in fraction IV were still left in solution at this concentration but disappeared completely if the $AmSO_4$ saturation was raised. No antigens were left in solution at $AmSO_4$ saturations higher than 75 per cent. When the antisera were absorbed with antigen solutions, fractionated as described above, and finally after the absorption were allowed to react with complete antigens solutions, marked serological differences between the fractions I–IV could be observed (diagram 3).

Besides the experiments reported above a series of experiments was undertaken with extracts, made in 2 M sodium chloride from the residue after the usual extraction with 0.14 M saline. The solutions were dialyzed and treated in the usual manner. These extracts contained at least one soluble antigen, not extractable with sodium chloride in lower concentration. This fact could also be proved by absorption experiments.

In another series of experiments the changes in the serological pattern during development were studied. The experiments showed, that the "spectrum" of saline soluble antigens is more or less the same for the unfertilized eggs and for the developmental stages studied. Most of the antigens are in common and of approximately the same concentration in extracts from all stages. Some quantitative differences seem, however, to exist. More about these will be reported in a subsequent publication. However, some changes of a more qualitative nature could be observed: in 48 hours plutei, at least three soluble antigens were found, which were absent in the extracts from unfertilized eggs (diagram 1: a, b, c). Thus after absorption of an antiserum against 48 hours plutei with extracts from unfertilized eggs, still three lines were obtained in the reaction with extracts from 48 hours plutei (Fig. 3). These antigens seem to belong to fraction I and II. Apparently they are not present or at most they are present in very small quantities only, in the insoluble residues from the egg. Otherwise, antibodies should have been formed at immunisation. Their appearence in solution was first detected in the extracts from 30 hours old gastrulae stages.

Besides the extracts mentioned above, 0.14 M saline extracts from lyophilized sperm and from jelly coat, precipitated by $AmSO_4$, were tested with antisera against eggs and developmental stages. As can be seen from Fig. 4, extracts from the sperm have one antigen fraction in common with the egg. The same is the case with the jelly solution (Fig. 5). This could also be proved by testing antisera against sperm and jelly coat with their homologous antigens and with egg extracts. In those cases, only 1–2 lines, belonging to fraction II, were obtained with the egg solutions. In addition neither the sperm extract nor the jelly solution seemed to contain antigens, not occurring in the egg.

In connection with a recently started work in collaboration with Drs. C. and D. Harding on the antigens of some sea urchin hybrids, eggs and developmental stages of the species *Psammechinus microtuberculatus* and *Arbacia lixula* have also been investigated. The experiments showed, that the antigenic set up of the two species *Paracentrotus lividus* and *Psammechinus microtuberculatus* is very similar. Both contain several antigens in common. Anyhow, in each species antigens exist which are lacking in the other species (Fig. 6). On the other hand, almost no similarity was found between the soluble antigens from those two species and from *Arbacia lixula* (Fig. 7). Only a very weak cross reaction could be obtained in the heterologous reactions between extracts and antisera from those species. The results of this investigation will be published later.

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CHANGE OF DISTRIBUTION OF MITOCHONDRIA IN ANIMAL HALVES OF SEA URCHIN EGGS BY THE ACTION OF MICROMERES

P. LENICQUE, S. HÖRSTADIUS and T. GUSTAFSON

The Wenner-Gren Institute for Experimental Biology, University of Stockholm, and the Zoological Institute, University of Uppsala, Sweden

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Isolated meridional halves of sea urchin eggs give rise to typical dwarf pluteus larvae but the differentiation of an isolated animal half does not coincide with that of the same material in a whole egg. As a rule the apical tuft becomes more or less enlarged, and neither stomodaeum nor ciliated band are developed, owing to a lack of vegetal properties. It was further found, that implantation into animal halves of the strongest vegetal material, the micromeres, leads to formation of an archenteron and development into a perfect pluteus larva of half the usual size (4, 5, 6, 7).

These phenomena have been explained by assuming the existence of an animal and vegetal gradient in the sea urchin egg, both overlapping throughout the whole egg. These gradients represent the intensity of two different mutually antagonistic types of metabolism. A certain proportion between these two types is necessary for a typical development (10).

Child (1) has shown a differential reduction of methylene blue and Janus green in the early development of sea urchin eggs. In the late blastula and during gastrulation there is a reduction gradient from the vegetal towards the animal pole coexistent with a weaker gradient from the animal pole. Hörstadius (8) studied the reduction gradients in animal and vegetal halves and in animal halves with implanted micromeres. In isolated vegetal halves we hardly ever find an animal gradient. In animal halves the animal gradient is comparatively much stronger than in the normal egg, and the vegetal gradient is entirely lacking. This illustrates how the vegetal forces normally suppress the animal to a certain extent. Relieved from the vegetal influence the animal properties assert themselves in a much stronger way. If four micromeres are implanted into an animal half, a vegetal gradient is induced and the animal one restricted to such an extent that the gradient system now resembles that of a normal egg at the same stage.

As to the physiological basis for the gradients Lindahl (9) has suggested that the animal principle is connected with carbohydrate metabolism, whereas the vegetal principle in later stages might be characterized by a breakdown of proteins. Gustafson (2) stresses that mitochondria contain a series of enzymes involved in protein synthesis, e.g. of fibrous proteins active in cell-stretching and in the formation of the apical tuft. The animal metabolic type favours the development of mitochondria, whereas the vegetal type produces inhibitors for mitochondrial development. In a normal late blastula or early gastrula the mitochondria thus decrease in number from the animal towards the vegetal pole. In corresponding stages of eggs animalized by iodosobenzoic acid the distribution is more uniform, due to the weakening of the vegetal metabolism. In Li treated larvae the gradient is depressed, especially in more vegetal regions.

The scope of the present paper is to study whether, in analogy with the induction and inhibition of reduction gradients, differences in the distribution of mitochondria appear when comparing whole eggs, isolated animal and vegetal halves, as well as animal halves with implanted micromeres.

MATERIAL AND METHODS

The experiments were performed in the summer of 1952 at the Kristineberg Zoological Station on eggs of *Psammechinus miliaris*.

The counts were made at the beginning of gastrulation respectively at corresponding stages. Shortly before the counts, the eggs were stained for a few minutes in Nile blue sulphate and placed under a cover slip. The right orientation was achieved by moving the cover slip slightly. The counts were made with the aid of an ordinary microscope of highest quality, and by inserting a network of 10 × 10 squares in the ocular. The slide was moved so that the different levels along the egg axis could be studied from the animal towards the vegetal pole. For further methodological details see Gustafson and Lenicque ((3) p. 252–254).

The curves consist of the means of several experiments, the number of which is indicated together with the results.

RESULTS

The mitochondrial curve of a differentiating isolated animal egg half (Fig. 1 an., 8 cases) differs markedly from the corresponding vegetal egg half (Fig. 1 veg., 6 cases). The difference does not only concern the level of the curves, but also the pattern of mitochondrial distribution.

Implantation of micromeres into an animal half converts the mitochondrial curve into a more vegetal type (Fig. 1). The effect is clearly a function of the number of micromeres implanted. Thus four micromeres induce a transition into a curve (Fig. 1 an. +4, 5 cases) characteristic of a normal larva (Fig. 1, control), whereas the 8 micromere curve (an. +8, 3 cases) is very similar to that of a differentiating isolated vegetal half (veg., 6 cases). In the most vegetal part of the germ full response may, however, already by obtained after the implantation of four micromeres.

The simple mitochondrial pattern of a mesenchyme blastula gradually transforms into a more complicated one as development proceeds (3). The present studies show that this also applies to the development of an isolated vegetal half (Fig. 2). Analogously, the simple mitochondrial gradient of a larva animalized by iodosobenzoic acid, which persists in advanced stages of development, is closely similar to that of an advanced isolated animal half (Fig. 3).

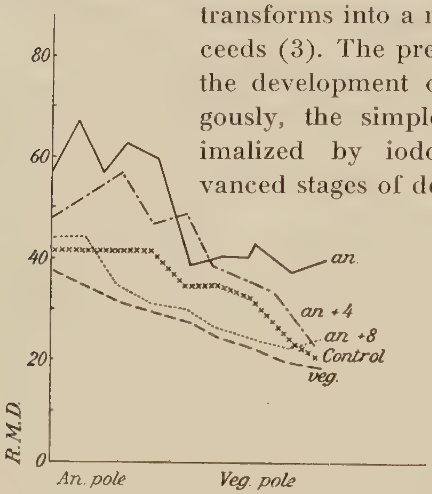

Fig. 1. Curves showing the mitochondrial distribution along the animal-vegetal axis in late blastulae — early gastrulae of sea urchins (Control), in isolated animal (an.) and vegetal halves (veg.) as well as in animal halves with 4 (an. + 4) or 8 implanted micromeres (an. + 8). R. M. D., relative mitochondrial density.

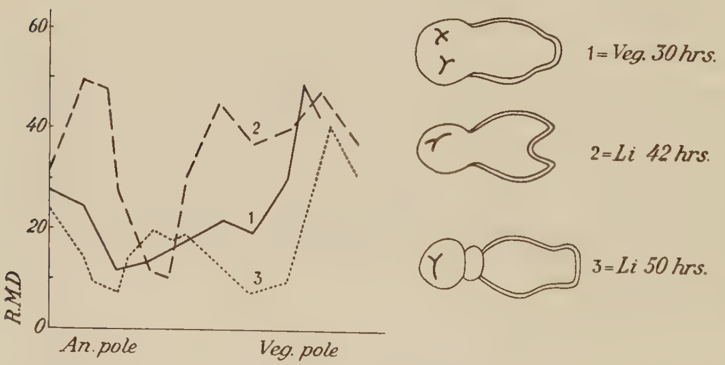

Fig. 2. Curves showing the mitochondrial distribution in 1) an isolated, exogastrulated vegetal half at an age of 30 hrs.; 2) a whole egg treated with Li, age 42 hrs.; 3) Li treated larva, age 50 hrs. 2 and 3 from Gustafson and Lenicque (3).

DISCUSSION

This study could be extended to comprise animal halves vegetalized by lithium, vegetal halves animalized with iodosobenzoic acid and the long series of isolated and in various ways recombined egg fragments studied by Hörstadius (5). The present results together with those of Gustafson and Lenicque (3) are, however, adequate for demonstrating the equivalence in mitochondrial pattern of chemically and operatively altered larvae. The mitochondrial pattern is thus characteristic for the morphological type, irrespective of the way it was obtained. The results also confirm earlier observations (3) that the size of the apical tuft and the density of the mitochondrial population both vary as a function of the relative vegetal strength. This is in conformity with the suggestion made by Gustafson and Lenicque that there is a connection between the mitochondrial processes and the production of the fibrous structure proteins of the apical tuft. Furthermore, the variation of the mitochondrial gradient is a parallel to the above mentioned variation of the redox-gradient system in corresponding fragments (8).

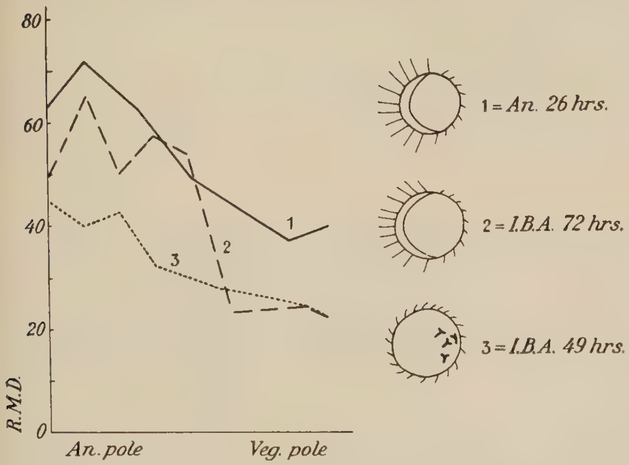

Fig. 3. Curves showing the mitochondrial distribution in 1) an isolated animal half, age 26 hrs.; 2) a whole egg treated with iodosobenzoic acid, age 72 hrs.; 3) the same treatment, age 49 hrs. 2 and 3 from Gustafson and Lenicque (3).

SUMMARY

The mitochondrial pattern of an isolated animal egg half agrees with that of a larva animalized by iodosobenzoic acid, while the pattern of an isolated vegetal half is consistent with that of a lithium vegetalized larva. Implantation of micromeres into an animal half shifts the pattern into that of a normal or a vegetalized larva. The strength of the effect is a function of the number of micromeres implanted.

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STUDIES ON THE HEMOGLOBIN FORMATION DURING REGENERATIVE ERYTHROPOIESIS

E. HAMMARSTEN, B. THORELL, S. ÅQVIST, N. ELIASSON and L. ÅKERMAN

Department of Biochemistry and Department of Pathology, Karolinska Institutet, Stockholm, Sweden

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THE hemoglobin synthesis is a process that occurs parallel with a cellular development, the erythropoiesis. The relationship of the hemoglobin formation to the growth and division processes during this development has been demonstrated by microspectrographic analyses of single erythroblasts at different stages of maturation. Fig. 1 shows the quantitative changes in the dominant substances of the erythroblast. The concentration of the cytoplasmic nucleotides is high in the early stages prior to and during the main formation of cells by growth and divisions. Parallel with the erythroblastic maturation the cytoplasmic nucleotide concentration decreases. When a relatively low level has been reached, the formation of hemoglobin (measured as light absorption in the Soret band region) takes place.

To reveal the significance of these data with respect to the synthesis of red cell substance, it seems necessary to investigate the metabolic behaviour of the different cells in the erythropoietic sequence. In the present work this was done by combining cytochemical methods with the tracer technique.

The primary questions were:

1) Does the relatively high nucleotide concentration in the immature erythroblasts correspond to a high metabolic activity, possibly related to synthesis of cellular proteins?

2) Is the globin part of hemoglobin also synthetized in the immature growing erythroblasts, so that the endocellular formation of hemoglobin during erythropoiesis is effected at least in two steps — formation of globin in the presence of nucleic acids during the earlier phases of growth, and synthesis of hemin during the later phases of maturation?

EXPERIMENTAL

The principle for the experiments was to separate cells of the different stages of erythropoiesis in amounts sufficient for macrochemical preparations and analyses, so that the incorporation of isotope in the cellular components could be studied. In order to correlate the turnover data with the growth and maturation processes, cytochemical and cytological analyses of the different samples were made.

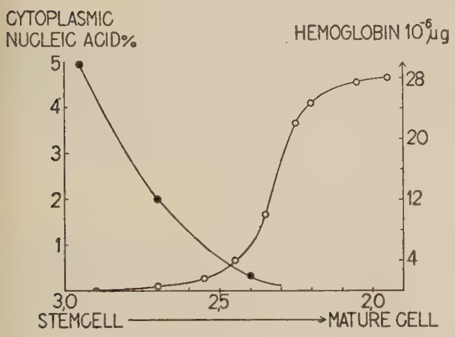

Fig. 1.

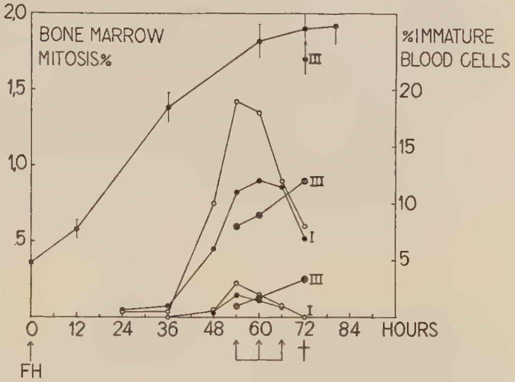

Fig. 2.

Fig. 1. Survey of the changes in cytoplasmic composition during erythropoiesis. Cytoplasmic polynucleotides – ● – ● –, total amount of hemoglobin in the cell – ○ – ○ –. Abscissa: logarithm of cellular volume. The values are plotted from ref. 13.

Fig. 2. Survey of some changes in the blood and bone marrow after intramuscular injection of a one per cent solution of phenylhydrazine hydrochloride at time 0 (FH). The percentage of immature blood cells is calculated according to their content of hemoglobin. The upper group of curves represents cells with approximately $5 \cdot 10^{-6}$ µg hemoglobin, the lower group represents cells with no detectable hemoglobin. The values are from experiments I b and III in Table III. The bone-marrow mitosis frequencies (– ┿ – ┿ –) are estimated on individual hens. They are measured on stained paraffin-sections and calculated according to the Poisson-distribution. The three arrows indicate the time of isotope injection in experiments I and III.

Young hens weighing about 1.5 kg were used in the experiments. They were all obtained from an inbred strain of White Leghorn (Edo). Before and during the experiments they had free access to a standardized chicken food (Fors). Their consumption of food was roughly controlled and did not change much under the experimental conditions. A severe anemia was produced by phenylhydrazinehemolysis (50 mg per kg of body weight), after which treatment the hen starts a regeneration of red cells (Table I). During the regeneration the bone marrow becomes filled with immature growing and dividing cells. The later phases of erythropoiesis, the hemoglobin forming cells, are thrown out into the blood stream (Fig. 2). Fig. 3 shows a photomicrograph of the normal hen bone marrow; Fig. 4, 12 hours after the injection of phenylhydrazine, Fig. 5, 72 hours after the anemia was produced, and Fig. 6 peripheral blood after the same period.

The cytochemical analyses of the bone marrow and blood cells during the regeneration were made by the microspectrographic technique described earlier (13, 14). Paraffin-sections and smears stained with acid and basic dyes were used as complement. Fig. 7 shows the composition of bone marrow and peripheral blood in a normal hen. The different developmental stages of erythropoiesis are divided into groups on the basis of the concentration of cytoplasmic nucleic acid and hemoglobin.[1] The mitosis frequency in this bone marrow is approximately. 2 per cent.

TABLE I

Red blood cell counts (millions/mm³) after phenylhydrazine hemolysis in some typical experiments

Hours	Hen: 1.	2.	3.
0	5.70	5.04	5.16
12	1.75	–	–
24	1.66	2.42	2.02
36	1.68	2.06	1.75
48	1.85	1.70	1.83
60	2.05	1.45	–
80	–	1.54	2.00
100	–	1.91	–

The error at random in the tabulated counts lies around 3 per cent.

Fig. 8 shows the composition of bone marrow and peripheral blood 72 hours after the anemia was produced. It will be seen that the erythropoiesis is divided by the bone marrow - blood boundary into two parts. The growing, dividing cells occur in the bone marrow, and the hemoglobin formation takes place in the erythroblasts in the blood stream. The mitosis frequency in this bone marrow is approximately 2 per cent. There are no mitoses in the erythroblasts in the blood.

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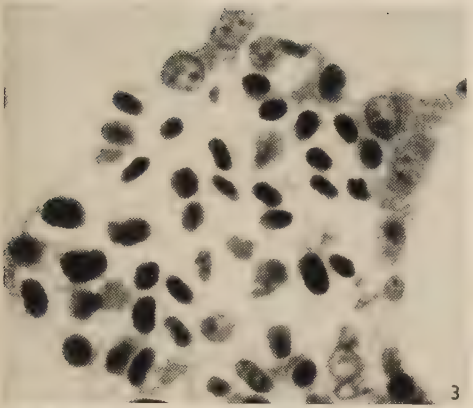
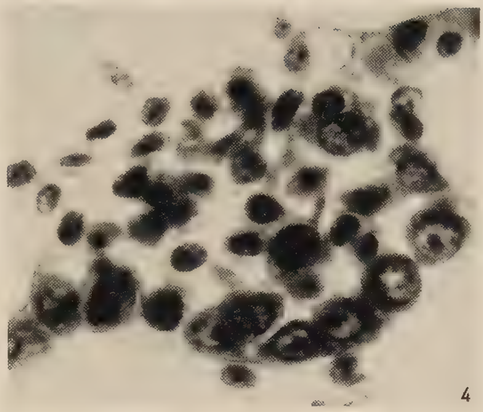
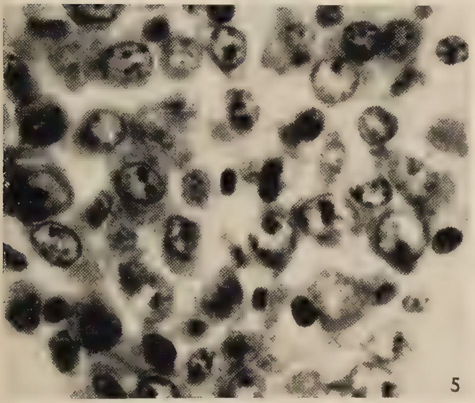
[1] In the following as in earlier publications (12, 13, 14) the concentrations measured microspectrographically are defined according to Lambert-Beers law as $c = \frac{E}{k \cdot d}$, where E is the extinction ($\log \frac{I_0}{I_t}$), k the extinction coefficient and d the thickness of the cell at the measured point. The points are in those parts of the cytoplasm where a reasonably good optical homogeneity allows a determination of the light absorption. In the case of erythroblasts and also immature myeloid cells in a fresh bone marrow suspension such points can mostly be chosen arbitrarily. The values obtained are thus representative for the average cytoplasmic c. For details of the measurement procedures, see ((13), p. 20–44 and (14)).







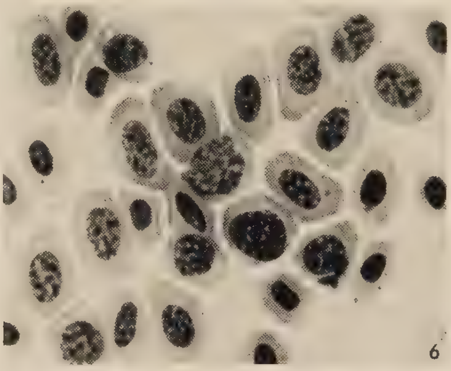


Fig. 3. Photomicrograph taken at 2570 Å of an unstained section from normal hen bone marrow, showing a typical sinusoid. Objective: quartzmonochromat 1.6 mm, condenser aperture: 2 × 30°, magnification 1100 ×.
Fig. 4. Photomicrograph of a hen bone marrow 12 hours after the phenylhydrazine hemolysis. Optical data as in Fig. 3. The peripheral cells of the sinusoid show signs of growth activity.
Fig. 5. Hen bone marrow 72 hours after the phenylhydrazine hemolysis. Optical data as in Fig. 3. The sinusoides are distended with immature growing and dividing erythroblasts.
Fig. 6. Stained blood-smear from a hen 72 hours after the phenylhydrazine hemolysis, showing a high content of immature cells.

A general picture of the changes in the bone marrow polynucleotides during different stages of regeneration is shown in Table II. The ratio of ribose to desoxyribose polynucleotides (as nitrogen per dry weight) increases parallel to increasing growth activity and the content of immature cells, in conformity with the cytochemical data (2, 15).

Under the afore-described experimental conditions the hen was given glycine labeled with N^{15} intraperitoneally. Glycine was chosen because it is directly incorporated into the purines of the nucleic acids, the proteins and the hemin (3, 5, 11). After a certain lapse of time the hen was sacrificed by exsanguination, and the different substances in the bone marrow and blood cells were separated, degraded, and analyzed.

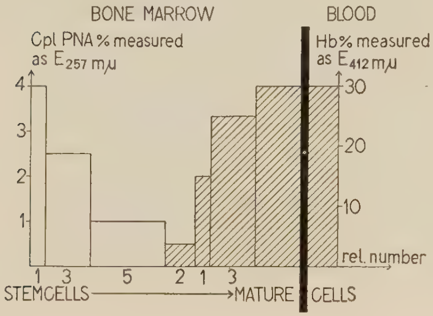


Fig. 7.

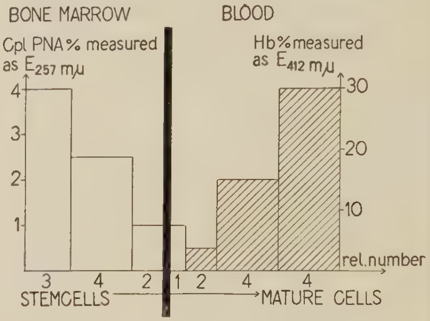


Fig. 8.

Fig. 7. Survey of the distribution of the different erythropoietic stages in bone marrow and blood of a normal hen. The scheme is based on measurements of 200 cells. The empty columns represent cells with measurable cytoplasmic nucleotides, the striped columns represent hemoglobin-containing cells. The relative magnitude of each group is marked on the abscissa. The coarse, vertical line denotes the "boundary" between the bone-marrow and the peripheral blood.
Fig. 8. The distribution of the different erythropoietic stages in bone-marrow and peripheral blood of a hen 72 hours after the phenylhydrazine hemolysis. The same denotations as in Fig. 7. (In Figs. 7 and 8 the extrasinusoidal myeloid cells are not shown. These cells do not undergo any significant changes during our experimental conditions.)

Table II

Hours after phenylhydrazine hemolysis	Bone marrow mitosis frequency in per cent	Extractable ribose-polynucleotides; Nitrogen μg per 100 mg dry tissue	Extractable desoxyribose-polynucleotides	$\frac{\text{RNA}}{\text{DNA}}$ per cent
0	0.36 ± 0.08	3.60	7.59	47
12	0.58 ± 0.12	5.61	12.05	47
36	1.38 ± 0.19	6.28	12.10	52
60	1.82 ± 0.22	8.01	13.72	58

The polynucleotides were extracted by Hammarsten's (4) method, and the purines fractionated (4, 10). The proteins were prepared and the amino acids separated (1). Hemin was crystallized and recrystallized by the method of Nencki and Zaleski (8). The globin was prepared from a hemolysate according to the procedure described by Steudel (12).

In each experiment small samples of blood were frequently collected for counting and cytological analysis. At the end of the experiment samples of bone-marrow were also removed for cytological investigations.

Table III shows a survey of the four different experiments reported here. The experiments I, II and III were made mainly for the guidance of how to plan experiment IV A, B and C upon which we are laying the chief stress.

TABLE III

Experiment	Time of isotope injection and sacrifice. Hours after hemolysis		Duration of isotope period in hours	Dosage of labeled glycine[1]
	Isotope injection	†		
I a	54, 60, 66	72	18	2 g/kg
b	54, 60, 66	72	18	»
II a	6, 12, 18	60	54	2 g/kg[2]
b	6, 12, 18	60	54	4 g/kg
III	54, 60, 66	72	18	2 g/kg
IV A	40, 42, 44	46	6	2 g/kg
B	74, 76, 78	80	6	»
C	43, 45, 47	81	38	»

It will be seen that the intentional differences between these experiments are the stage of blood-cell regeneration (time after hemolysis) at injection of labeled glycine and the length of the isotope-period. By means of these variations we aimed at investigating the successive phases during the differentiation processes of the immature blood cells.

DISCUSSION OF RESULTS

Table IV gives a survey of the turnover data in normal hens (pooled samples from four animals) compared with those in hens regenerating 54 to 72 hours after phenylhydrazine-hemolysis. The cytochemical and hematological data from these hens are presented in Figs 2, 3, 5, 6, 7 and 8. The incorporation of N^{15} in the desoxyribose polynucleotides is higher in the regenerating than in the normal bone marrow. This can be correlated with the higher mitosis frequency in the former, in agreement with the findings in other material (3, 6). The incorporation of N^{15} in the ribosepolynucleotides (RNA), on the other hand, indicates that the synthesis of this substance is not much higher

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[1] 31.0 atom per cent excess of N^{15}.

[2] Besides N^{15} also labeled with C^{14} in the carboxyl group (30 000 counts/min).

Table IV

Experiment I a and b. Atom per cent excess N^{15}.

	Bone marrow					Erythrocytes						
	DNA		RNA		TCA-ppt[1]	Nuclei[2]					Hemolysate[2]	
	ad	gu	ad	gu		DNA		RNA		TCA-ppt[1]	Hemin[3]	Globin
						ad	gu	ad	gu			
Normal hen (a)	.69	.66	1.60	1.55	.55	.084	.105	.016	.044	.034	.044	.004
Regenerating hen (b)	1.82	1.81	1.89	2.32	.937	.173	.174	.217	.20	.201	4.23	–

[1] Proteins of the precipitate with hot trichloracetic acid after the polynucleotide-extraction.

[2] Prepared by saponin hemolysis. The nuclei are freed from the cell membranes by several washings with physiological saline.

[3] No corrections have been made for unlabeled mature cells, already present in the peripheral blood at the beginning of the experiment. We think, however, that this does not significantly affect the results.

Table V

Experiment II a and b. Atom per cent excess N^{15}.

	Erythrocytes									Bone marrow			
	Hemolysate				Nuclei					DNA		RNA	
	Hemin	Globin	Glycine isol. from globin	Hemin/ Glycine	DNA		RNA		TCA ppt	ad	gu	ad	gu
					ad	gu	ad	gu					
Exp. II a (2 g labeled glycine/kg)	2.040	.256	2.601	.78	.712	.651	–	–	.208	–	–	–	–
Exp. II b (4 g labeled glycine/kg)	1.953	.841	7.20	.27	1.613	1.621	1.532	1.617	–	1.803	1.710	2.433	2.737

in regenerating bone marrow than it is in normal bone marrow. With reference to an investigation on regenerating liver (3), we think that this small difference in RNA levels (concentration of RNA and isotope incorporation) means that the need for RNA-polynucleotides required in the protein synthesis in regenerating bone marrow is almost fulfilled at the RNA-concentration reached in the growing and dividing cells at this stage of regeneration and that it is the same as in the most immature RNA-containing cells of the normal bone marrow (cf. Figs. 7 and 8).

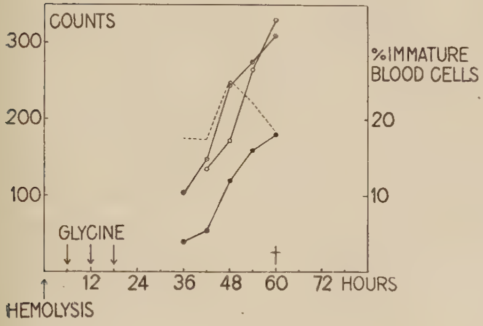

Fig. 9.

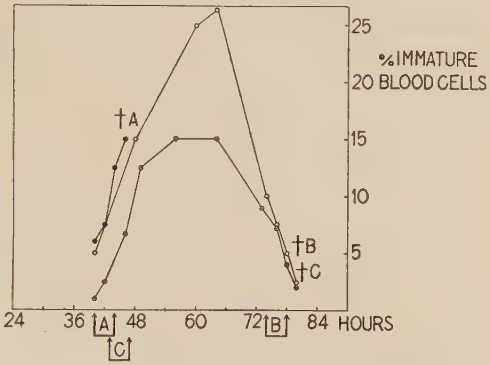

Fig. 10.

Fig. 9. Survey of experiment II a. The glycine used was besides N^{15} also labeled with C^{14} in the carboxyl group (30 000 counts/min.). Curve — — — — represents counts per two minutes of .5 ml plasma dried on a disc 20 mm in diameter. Curve – ○ – ○ – represents counts per two minutes of 15 mg dry weight of washed red cells on a similar disc. Curve – ⊙ – ⊙ – is counts per two minutes of 15 mg of dry red cell nuclei prepared after saponin-hemolysis and five washings with physiological saline solution, dried on a similar disc as above. The background was eleven counts per two minutes. The plotted values may be regarded as indicating the relative increase of isotopes in the cells of the peripheral blood. Curve – ● – ● – represents the increase of immature cells in the peripheral blood.

Fig. 10. Survey of experiment IV. Curve – ● – ● – represents immature cells in the peripheral blood of hen A, – ⊙ – ⊙ – hen B and – ○ – ○ – hen C. On the abscissa is plotted the time after phenylhydrazine hemolysis. The arrows indicate the first and last injection of labeled glycine in each hen.

The values of the amino acids in the bone marrow proteins have been presented and discussed (3).

The normal mature blood-cells show very low incorporation of N^{15} in the analysed substances, partly because of the relatively short duration of the isotope-period cf. (11). As may be expected according to the cytochemical data, there is a considerable synthesis of hemin in the regenerating blood cells. Unfortunately, the globin-preparation from this experiment was lost in an accident. The incorporation of N^{15} in the polynucleotides shows an increase in the regenerating cells, although this increase was not of the magnitude of that displayed by hemin.

The experiment II a and b, illustrated in Table V and Fig. 9, was designed to reveal how long it takes before a labeled bone-marrow cell enters the blood stream and also to study the effect of the glycine dose on the relationship between the incorporation values, in the first hand hemin/globin. The animals were injected very soon (six hours) after the phenylhydrazine-hemolysis had been produced. After 36 hours samples of blood were collected six-hourly, and the animals were sacrificed at 60 hours (see Fig. 9). Between 36 and 48 hours after the injection of isotopes, the isotope content (C^{14}) in the blood-cells shows an increase. The concentration of isotope (N^{15}) reaches a high level especially in the proteins and polynucleotides. A twice as large dose of labeled glycine gives a relatively higher incorporation in the globin than in the hemin. This may indicate that the isotope, when given during the very earliest phases of regeneration (6 18 hours), is mainly used in the synthesis of polynucleotides and globin.

In the next experiment (III) we used a hen which during the isotope-period increased its content of the most immature blood-cells from 8 to 12 per cent (Table VI, Fig. 2). Analysis of these cells, many of which were thus coming directly from the actively growing bone-marrow, showed a relatively high incorporation of isotopes in the proteins and polynucleotides. The quotient hemin/glycine isolated from globin was as low as 0.28.

On the basis of the obtained results we designed a final experiment including three hens, each of which was to be given the same amount of labeled glycine, but during different stages of blood-cell maturation (Fig. 10, Table VII). The first hen (A) was given isotopes during a period when the amount of immature cells in the blood-stream was increasing. Newly formed, immature cells were thus rapidly thrown out from the bone marrow. Six hours later the animal was sacrificed and the isotope concentration in the blood-cell substances analysed. The second hen (B) was given labeled glycine when the immature blood cells were decreasing in amount, i.e., during their final maturation. She was also killed six hours later. The third hen (C) received isotopes during a period, when her immature blood-cells were increasing, but was not sacrificed until 38 hours later when the blood-cells were in the process of final maturation. (See Fig. 10.) It will be seen in Table VII that in the first hen the globin synthesis predominates in relation to the hemin synthesis. In the second hen the condition is reversed; the hemin production predominates in the blood-cells undergoing their final maturation.

TABLE VI

Experiment III. Atom per cent excess N^{15}.

Erythrocytes											Bone marrow			
Hemolysate					Nuclei						DNA	RNA		TCA ppt
					DNA		RNA		TCA ppt					
Hemin	Globin	Glycine isol. from globin	Hemin/ Glycine	RNA adenine	ad	gu	ad	gu			ad	ad	gu	
1.035	.407	3.724	.28	1.481	.220	.688	.283	.631	.239		1.2	1.594	2.001	1.041

TABLE VII

Experiment IV A, B and C. Atom per cent excess N^{15}.

	Erythrocytes									Bone marrow	
	Hemolysate					Nuclei				RNA	TCA ppt
						DNA		RNA	TCA ppt		
	Hemin	Globin	Glycine isol. from globin	Hemin/ Glycine	RNA adenine	ad	gu	ad		Adenine	
A	1.177	.209	1.627	.72	.287	.016	.030	.157	.082	1.935	.691
B	1.558	.138	.956	1.63	.014	.015	.057	.180	.135	1.152	.773
C	.465	.537	5.29	.09	—[1]	—	—	—	—	—	—

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[1] Amounts too small for analysis.

The third hen (C) shows a markedly dominating incorporation in the globin, as could be expected in view of the earlier experiments listed in Tables V and VI, indicating an earlier start of globin synthesis than of hemin synthesis. The level of N^{15}-concentration in glycine at the time of the hemin synthesis has been relatively low.

Although these results indicate that the maximum rate of the synthesis of globin precedes that of hemin during erythropoiesis, they do not necessarily contradict the observation by Neuberger (9), who found no significantly different ratio of isotope incorporation in protein/protoporphyrin of blood-cell samples taken at close intervals after injection of isotopes in normal rabbits. As shown by London *et. al.* (7) the globin of very late stages in erythropoiesis (reticulocytes) is still metabolically active. This activity may very well conceal the isotope incorporation at early stages of globin formation, when the renewal of the blood-cells occurs at a slow rate.

The incorporation of N^{15} in the cytoplasmic polynucleotides shows a marked difference in the hens A and B, contrary to the nuclear polynucleotides. We think that the difference in the cytoplasmic polynucleotides reflects the different intensity of actual globin synthesis (3, 5). If the renewal of nuclear substances is assumed to take place mainly in actively dividing cells, the similarity of the incorporation values for the nuclear fractions indicates that no significant difference in the number of cellular divisions exists in the actual stages of erythropoiesis. According to the cytological data no mitosis can be found in the immature cells of the peripheral blood and very few cells, labeled at the very early immature stages can be presumed to pass from the bone marrow into the blood stream during the short period of isotope incorporation (six hours) (cf. Fig. 9). So it may be concluded that the similarity of the incorporation values for the erythroblast nuclei in the blood of the two hens indicates an almost identical dosage of labeled glycine per immature blood cell in hens A and B.

SUMMARY

The relationship between the synthetic processes of the polynucleotides, the globin and the hemin during erythropoiesis has been studied, using glycine labeled with N^{15} as the tracer-precursor in regenerating blood-cells of the hen.

In normal and regenerating bone marrow the isotope contents in the purines from the desoxyribose-polynucleotides could be correlated to the mitotic activity.

A relationship existed between the isotope contents in the purines from the ribose-polynucleotides in the hemolysate of the immature blood-cells and the incorporation values for the globin.

The ratio of the incorporation values for globin and those for hemin isolated from blood cells at different levels of maturation indicated that during erythropoiesis under our experimental conditions the maximum rate of the synthesis of globin precedes that of hemin.

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ON A NORMALLY OCCURRING REDUCTION-DIVISION IN SOMATIC CELLS OF THE SEA URCHIN EMBRYO

P. E. LINDAHL

Institute of Zoophysiology, University of Uppsala, Sweden

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The fertilized sea urchin egg divides three times equally, the three successive planes of cleavage being at right angles to each other. The resulting eight-cell stage thus consists of four animal and four vegetal cells, all of the same size. At the fourth division, four small cells, the micromeres, are cut off around the vegetal pole, while the four cells of the animal hemisphere divide meridionally into equal cells. At the fifth division all cells except the micromeres divide equally, or very nearly so. In the micromeres, again, small cells are cut off around the vegetal pole, these cells being called the small micromeres of the thirty-two cell stage, or briefly the "small micromeres". The cleavage of the micromeres of the sixteen cell stage is delayed in relation to that of the other cells of this stage (8).

The small micromeres behave differently from the rest of the embryo (5) also in other respects. They do not flatten against their neighbouring cells and give the impression of being more resistant against deforming forces. In dark field illumination they appear optically empty and cannot be vitally stained by methylene blue or nile-blue sulphate because all the granules taking up these stains are extruded from that part of the cytoplasm which is to be cleaved off as small micromeres. They also show a stronger light refraction. In all these respects the small micromeres coincide with the polar bodies. All these similarities suggest a common mechanism for the formation of these two kinds of structures (6).

However, these formal coincidences may also be an expression of a deeper similarity, i.e. that the small micromeres would function as "somatic polar bodies", the division leading to their formation implying a reduction of the chromosome number.

To settle this question, two different species of sea urchins, *Paracentrotus lividus* (Lamarck) and *Strongylocentrotus droebachiensis* (O. F. Müller) were studied. Sections[1] of the relevant stages of *P. lividus* prepared at Naples in 1930 were examined. The diploid chromosome number of this species is known to be 36 (1). Several micromeres in different stages of division were observed. In early telophase, in which all the chromosomes of the small micromeres are very short and may often be found in one section, they could easily be counted. In two embryos the number was found to be 18. In two late anaphase stages the direction of sectioning was such as to permit the counting of the chromosomes of the two daughter groups, all chromosomes being situated in one section. Both groups contained 18 chromosomes. In several embryos with anaphase stages of the micromere division and the chromosomes in two different sections, the chromosome number of the two groups varied between 17 and 21, the higher numbers probably depending upon the fragmentation of some chromosomes at the sectioning. Many divisions were found in metaphase or very early anaphase with the chromosomes still not separated into two groups and with the direction of sectioning parallel to the axis of the spindle. In such cases the number of chromosomes could not be counted exactly, but were estimated to fall between 32 and 40. It is thus obvious that the division of the micromeres of the sixteen cell stage implies a reduction of the number of chromosomes to half.

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[1] All sections referred to are 4–5 μ thick, stained with Heidenhain iron hematoxylin and prepared from material fixed in Bouin's mixture.

The *Strongylocentrotus*-material was obtained on the Swedish West Coast. Here this species lives under non-optimal conditions, which often results in an irregular cleavage and death before gastrulation. For the preparations studied, eggs from females having about 90 per cent normal sixteen cell stages were selected.[1]

A look at some slides obviously revealed that also in this species the division of the micromeres yields haploid nuclei. However, as far as this writer has been able to ascertain, its chromosome number is not known earlier. Counts were made in anaphase stages of mesomeres, macromeres, and micromeres. Counting the chromosomes of one and the same group from two sections may give somewhat too high values because of a fragmentation of a number of chromosomes. On the other hand, some of the chromosomes may easily escape observation, especially when counts are made in diploid groups where the chromosomes are very crowded and found in several layers hiding each other. Thus we will have to expect that counts obtained from haploid chromosome groups will be too high, and those from diploid groups too low. The haploid number of chromosomes was calculated from the 33 observations of haploid and diploid numbers presented below.

The number obtained is 20.2, which agrees well with that from an equatorial plate observed in polar view in a micromere (Fig. 1). Here the chromo-

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[1] Since this was written hundreds of *Paracentrotus*-micromeres in anaphase have been investigated in squash preparations. The two chromosome groups always were found to contain the haploid number.

		Haploid							Diploid					
Numbers of chromosomes		18	19	20	21	22	23	25	37	38	49	40	41	42
Frequences of observations		3	2	4	1	3	3	1	2	4	3	3	1	3
Number of observations	in mesomeres								7					
	in macromeres								8					
	in micromeres	17							1[1]					
	total	17							16					

somes are arranged in one layer. They could easily be counted and the number was found to be 40. As several of them are situated with their longitudinal axis not deviating much from the optical axis of the microscope, they appear perspectively shortened. In this plate, as in the other two of the three sister micromeres studied in this embryo, a pairing of the chromosomes is taking place, being equivalent to the "secondary pairing" frequently observed in mitosis in *Diptera* and polyploid plants and in meiosis in polyploid plants (for literature cf. (3)). Also in *Paracentrotus* a secondary chromosome pairing was observed in some micromeres. A corresponding pairing could not be revealed in metaphase plates of macromeres or mesomeres of *Strongylocentrotus droebachiensis* except for single chromosomes, and the same holds true with regard to the *Paracentrotus* material (cf. also Baltzer's figures (1)). So far, my present observations suggest that the secondary pairing is limited to the reduction-divisions in the micromeres. However, this problem will be reinvestigated by a more suitable method.

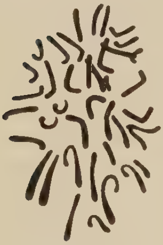

Fig. 1.

The functional similarity between polar bodies and small micromeres is further stressed by the fact that the latter, long after their formation, do not really join into the epithelial arrangement of the blastula·wall (6). As a matter of fact it has always been presupposed, but never proved that the small micromeres participate in the formation of the embryo.

According to Berger (cf. (2)) somatic reduction divisions occur in polyploid cells of the pupal ileum of *Culex pipiens* during metamorphosis. However, such divisions leading to *haploid* nuclei have apparently not been observed up to now in animals. In plants such divisions are known to occur spontaneously and have also been experimentally induced (for literature, cf. (2)).

The micromeres and their descendants have very important functions. During the blastula stage this material, having the most vegetal properties of the embryo, has a high potency in restraining the tendencies of extension of animal differentiations and when transplanted into isolated animal halves, in inducing vegetal differentiations (4). It is interesting to what degree these functional qualities are dependent upon the haploid chromosome number.

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[1] Equatorial plate

Later the descendants of the micromeres invade the blastocoel as skeleton-forming cells. In this connection it is a remarkable fact that in the early pluteus stage the nuclei of these cells do not, as might be expected, distinguish themselves by a striking smallness. In this stage various organs are characterized by nuclei of different sizes, those of the skeleton-forming cells falling near the mean of the extremes, i.e. the nuclei of the coelomic rudiment and those of the most dorsal ectoderm (7). Obviously, other factors than the chromosome numbers determine the nuclear size in the pluteus stage. Possibly a reconstitution of the diploid chromosome number occurs. However, this not being the case in the intermediate time, the skeleton substance is produced by haploid cells. Again the question arises as to what extent this function is dependent upon the haploid state.

In single *Strongylocentrotus* embryos abnormalities as to the chromosome numbers were observed. Haploid chromosome groups were found in dividing macromeres and even in mesomeres, and in one micromere division in anaphase the two chromosome groups appeared to be diploid. Such disturbances may be one of the causes why this material fails to develop normally. However, these observations demonstrate that in the sixteen cell stage the determination of a division to occur as a mitosis or as a somatic reduction-division is rather labile. Under the presumption that the haploidic state of the micromere descendants is an important factor in their power of influencing the determination of the primary germ layers, this lability may be of fundamental significance when the boundaries of these layers are displaced by chemical treatment. An experimental analysis ot these and related problems is in progress.

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DIE WIRKUNG VON PERJODAT AUF DEN ZUSTAND DER PLASMAMEMBRAN DES SEEIGELEIS

G. KRISZAT

Wenner-Grens Institut für experimentelle Biologie, Stockholm, Schweden

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In früheren Untersuchungen (5, 8) konnte gezeigt werden, dass Seeigeleier bei Behandlung mit $6 \cdot 10^{-5}$–$9 \cdot 10^{-5}$ N Natriumperjodat eine Befruchtungsmembran ausbilden. Eine Anzahl von Eiern entwickeln sich danach bis zur ersten Zellteilung und vereinzelte bis zum 8-Zellenstadium. Bei Anwendung von $5 \cdot 10^{-5}$ N Perjodat vermindert sich die Anzahl der aktivierten Eier, jedoch weisen auch diejenigen, die keine Befruchtungsmembran ausbilden, eine Reihe von Veränderungen auf, aus denen hervorgeht, dass sie durch die Perjodatbehandlung in einen Zustand der ,,Präaktivierung" versetzt worden sind. So können z. B. unreife Eier, die sich überhaupt nicht oder nur sehr schlecht befruchten lassen, nach Behandlung mit Perjodat sehr leicht befruchtet werden. Ausserdem lassen sich die Eier mit hypertonischer NaCl-Lösung leichter aktivieren wenn sie vorher mit $5 \cdot 10^{-5}$ N Perjodat behandelt werden. Eine Anzahl von ihnen bilden dann nach Überführung in die hypertonische Lösung eine Befruchtungsmembran aus.

Der Umfang der Aktivierung ist vom Reifezustand der Eier und von der angewandten Perjodatkonzentration abhängig. Der Grad der Aktivierung zeigt sich im Zustand der Plasmamembran nach der Behandlung der Eier mit hypertonischer NaCl-Lösung. Nichtaktivierte Eier haben eine leicht faltige ((6) Abb. 2), präaktivierte eine glatte und vollaktivierte eine kantige Oberfläche ((6) Abb. 3), wobei die Letzteren eine Befruchtungsmembran ausbilden.

Eine Reihe von früheren Untersuchungen haben ergeben, dass nicht hochgereinigte Präparate von ,,Fucoidin", Heparin (1) und auch Gallerthüllensubstanz (10) in bestimmter Konzentration die Befruchtung und Membranausbildung in negativer Hinsicht beeinflussen. Von diesen erschien ,,Fucoidin" für Vergleiche mit Perjodat im Rahmen der vorliegenden Versuche besonderes geeignet. Mit Hilfe des Zentrifugenmikroskops ist es möglich, die verschiedenen Zustände der Eier durch Zentrifugieren zwischen Seewasser und einer isotonischen Rohrzuckerlösung zu untersuchen. Diese Methode ist zuerst von E. B. Harvey (2, 3) bei befruchteten und unbefruchteten Seeigeleiern angewendet worden. In der vorliegenden Arbeit erschien es lohnend, die gleiche Untersuchungsmethode bei den mit Perjodat behandelten Eiern zu benutzen und diese im zentrifugierten Zustand mit unbehandelten Kontrollen zu vergleichen, um aus dem Grad der Streckung der Eier während des Zentrifugierens Schlüsse auf den Zustand der Plasmamembran zu ziehen.

MATERIAL UND METHODE

Die Versuche erfolgten während des Sommers an der Zoologischen Station Kristineberg an der schwedischen Westküste. Es wurden Eier von *Psammechinus miliaris* verwendet, die in einem Seewasser mit einem Salzgehalt von ca. 25 pro mille gehalten wurden. Diese Konzentration entspricht annährend dem Medium der litoralen Form dieser Art. In allen Versuchen ist die Gallerthülle der Eier vorher entfernt worden. Für diesen Zweck wurden die Eier ca 10 Min. lang in einem mit HCl schwach angesäuertem Seewasser (pH 4,5) gewaschen. Die für die Versuche verwendeten Natriumperjodatlösungen hatten eine Konzentration von $5 \cdot 10^{-5}$–$9 \cdot 10^{-5}$ N. Für jeden Versuch kamen frisch bereitete Lösungen zur Anwendung, die durch Verdünnen einer 0,1 N wässrigen Natriumperjodat Stammlösung mit der entsprechenden Menge Seewasser hergestellt wurden (8). ,,Fucoidin" ist in einer Konzentration von 0,05 % bei einer Einwirkungszeit von 30 Min. verwendet worden. Es handelte sich um ein aus *Fucus vesiculosus* gewonnenes Präparat (1). Nach fortgesetzten Untersuchungen von Vasseur an dem hiesigen Laboratorium ist der wirksame Faktor nicht das reine Fucoidin sondern eine andere Substanz, die wahrscheinlich auch eine Polysaccharidkomponente enthält. Dieser im ,,Fucoidin" enthaltene Bestandteil, der die Rigidität des Kortex herabsetzt und die Befruchtungsfähigkeit hemmt, soll im Folgenden als ,,Hemmungsfaktor F" bezeichnet werden. Das reine Heparin ist sicher auch nicht wirksam, sondern nur Präparate davon, die gewisse Nebenkomponenten enthalten. Der wirksame Faktor soll hier im gleichen Zusammenhang wie oben ,,Hemmungsfaktor H" genannt werden, da ihm befruchtungshemmende Eigenschaften zukommen.

Für die Aktivierung der Eier genügte eine Perjodatbehandlung von einigen Minuten. Danach wurden die Eier in eine Mikroküvette übertragen und für die Dauer von 8 Minuten in einem Zentrifugenmikroskop (Fabrikat Struers, Kopenhagen) mit einer konstanten Zentrifugalkraft von 4350 g zentrifugiert. In der Mikroküvette waren die Eier zwischen Seewasser und einer isotonischen Rohrzuckerlösung suspendiert. Während des Zentrifugierens wurde die Streckung der Eier zwischen der Grenzschicht der beiden Lösungen mit einem Messokular bestimmt und mit der Streckung unbehandelter Kontrollen verglichen. Ausserdem wurde das gleiche Material in Seewasser mit einem Zusatz von 2 % Dextran in einer gewöhnlichen Zentrifuge unter Anwendung einer konstanten Zentrifugalkraft von 4460 g für die Dauer von 8 Min. zentrifugiert, um die Rigidität des Zytoplasmas durch die Messung der Schichtung der Plasmakomponenten zu bestimmen. (Nähere Angaben über Methodik siehe (9)). Im Zusammenhang mit den obenerwähnten Untersuchungen wurde das Verhalten der Eier nach Überführung in eine hypertonische Lösung beobachtet (0,6 ml. 2,5 N NaCl + 2 ml. Seewasser), um die dabei auftretende Form der Plasmolyse festzustellen.

ERGEBNISSE

Nach Behandlung der unbefruchteten Eier mit einer Konz. von $5 \cdot 10^{-5}$ N Perjodat zeigt sich beim Zentrifugieren ein deutlicher Unterschied zwischen Eiern, die nicht aktiviert und solchen, die präaktiviert oder vollaktiviert sind.

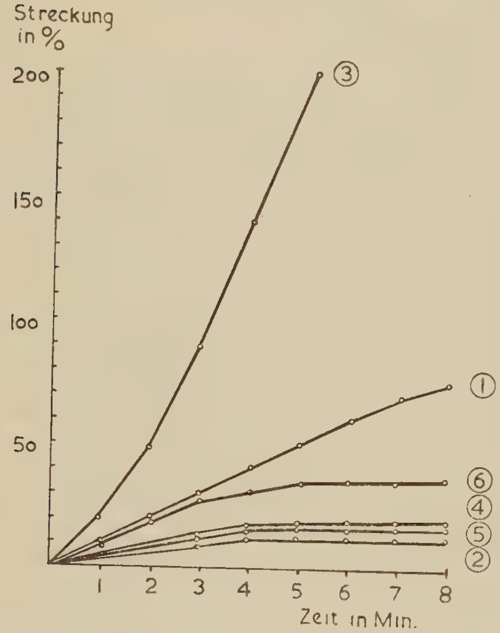

Abb. 1. Streckung der Eier im Zentrifugenmikroskop bei Anwendung einer konstanten Zentrifugalkraft während der Zeit von 8 Minuten. Die angegebene Streckung drückt die Abweichung vom Normaldurchmesser des ungestreckten, unbefruchteten Eis während des Zentrifugierens in % aus. 1. Unbefruchtete, unbehandelte Eier. 2. Mit 0,05 % „Fucoidin“ behandelte Eier. 3. Mit $5 \cdot 10^{5}$ N Natriumperjodat präaktivierte Eier. 4. Mit $9 \cdot 10^{-5}$ N Natriumperjodat vollaktivierte Eier, 10 Minuten nach der Bildung der Befruchtungsmembran. 5. Normale befruchtete Eier 10 Minuten nach der Befruchtung. 6. Mit $6 \cdot 10^{-5}$ N Natriumperjodat behandelte Eier, die vorher für 30 Min. mit 0.05 % „Fucoidin“ behandelt wurden.

Die nichtaktivierten Eier zeigen, wie aus der Abb. 1,1 hervorgeht, eine Verlängerung von ca 75 % über den Normaldurchmesser. Das entspricht dem bei den unbehandelten Kontrollen gemessenen Wert. Neben den Nichtaktivierten sieht man während des Zentrifugierens Eier, die sich ungewöhnlich stark strecken und bereits nach 4 Minuten eine Verlängerung von ca. 140 % aufweisen (Abb. 1,3). Es handelt sich hierbei um präaktivierte Eier, die keine Befruchtungsmembran ausbilden, die sich aber in ihrem Zustand durch die starke Streckung deutlich von den Nichtaktivierten unterscheiden. Die vollaktivierten Eier verhalten sich dagegen so wie normal Befruchtete und zeigen die gleiche Streckung wie diese (Abb. 1,4). Eine Unterscheidung der drei erwähnten Zustände ist auch mit Hilfe einer Hypertonie-Behandlung möglich, wobei die anfangs beschriebenen, verschiedenen Plasmolysetypen auftreten, die den unterschiedlichen Zustand der Plasmamembran von nichtaktivierten sowie prä- und vollaktivierten Eiern wiederspiegeln. Zentrifugiert man gleichzeitig unbehandelte und präaktivierte Eier in einer gewöhnlichen Zentrifuge für die Dauer von 8 Minuten, um den Verlauf der Schichtung im Zytoplasma zu vergleichen, so zeigen sich hier keine wesentliche Unterschiede zwischen beiden.

Mit Perjodat vollaktivierte Eier verhalten sich in Bezug auf die Schichtung der Plasmakomponenten so wie normal Befruchtete, wenn sie unter zeitlich gleichen Bedingungen miteinander verglichen werden. Man sieht in diesem Fall deutlich, dass etwa 10 Min. nach der Befruchtung die Rigidität des Zytoplasmas zunimmt. Während dieser Zeit zeigen sowohl die mit Perjodat behandelten als auch normal befruchtete Eier eine glatte Oberfläche nach Überführung in hypertonisches Seewasser. Etwa 15 Minuten nach der Bildung der Befruchtungsmembran kommt es in beiden Fällen zur Ausbildung einer kantigen Oberfläche bei Hypertoniebehandlung. Mit Perjodat vollaktivierte Eier zeigen also in Bezug auf das Verhalten der Plasmamembran und des Zytoplasmas, nach der Ausbildung der Befruchtungsmembran im wesentlichen eine gleichartige Periodizität wie normal befruchtete Eier.

Werden unbefruchtete Eier für 30 Minuten mit 0,05 % ,,Fucoidin" behandelt, so geht die Streckung der Eier im Zentrifugierversuch auf einen erheblich niedrigeren Wert zurück, als er bei unbehandelten Kontrollen gemessen wird (Abb. 1,2) Diese Wirkung des ,,Fucoidins" lässt sich durch anschliessende Perjodatbehandlung wieder vermindern (Abb. 1,6). Gleichzeitig wurde beobachtet, dass nach anschliessender Perjodatbehandlung die Rigidität des Zytoplasmas bei den mit ,,Fucoidin" vorbehandelten Eiern sinkt, dagegen zeigen die mit ,,Fucoidin" Behandelten eine wesentlich höhere Zytoplasmarigidität als unbehandelte Kontrollen.

BESPRECHUNG DER ERGEBNISSE

Es konnte gezeigt werden, dass neben den Eiern, die durch Perjodatbehandlung nicht aktiviert werden, auch noch solche auftreten, die zwar keine Befruchtungsmembran ausgebildet hatten, dabei aber Veränderungen aufwiesen, die erkennen liessen, dass sie sich nicht so verhielten wie normale unbefruchtete Eier. Sie wurden im Zentrifugenmikroskop ungewöhnlich lang gestreckt und hatten bei Hypertoniebehandlung im Gegensatz zu unbefruchteten Eiern eine glatte Oberfläche.

Es ist angenommen worden, dass das Perjodat auf einen Verbindungskomplex von Polysaccharid-Proteincharakter in der Oberfläche des Eies einwirkt und dass sich die Wirkung von dort aus in das Zytoplasma ausbreitet (10). Versuche (1) haben ergeben, dass bestimmte Komponenten in

Verbindungen von Polysaccharidcharakter, wie z. B. „Fucoidin" (12), Heparin (7), oder Gallerthüllensubstanz (7), in entsprechender Konzentration die Befruchtung und die Ausbildung einer normalen Befruchtungsmembran mehr oder weniger hemmen und dass diese Wirkungen mit Perjodat aufgehoben werden können. Sowohl das Verhalten der perjodatbehandelten Eier im Zentrifugenmikroskop, als auch in hypertonischen Lösungen erlaubt gewisse Schlussfolgerungen in bezug auf die Veränderungen, die sich in der Oberfläche des präaktivierten und des aktivierten Eies vollzogen haben. Man kann vermuten, dass bei der Präaktivierung durch schwache Perjodatwirkung eine Lösung bestimmter Bindungen in der Plasmamembran stattfindet und dass bei vollständiger Aktivierung eine Neu-Orientierung der submikroskopischen Partikel erfolgt. Für die erste Annahme spricht die starke Streckung präaktivierter Eier und das Fehlen einer kantigen Plasmolyse, für die Zweite der stärkere Widerstand aktivierter und befruchteter Eier beim Zentrifugieren und das veränderte Verhalten im Plasmolyseversuch.

Vermutlich wirken im reifen unbefruchteten Ei die Polysaccharid Komponenten wie eine Zementsubstanz, welche die submikroskopischen Fasern miteinander verkittet. Bei der Präaktivierung erfolgt primär eine Einwirkung auf diese Substanz. Durch Lösung einzelner Bindungen wird eine Auflockerung im submikroskopischen Fasergerüst der Grundsubstanz bewirkt, sodass eine Verschiebung und Neuorientierung der einzelnen Partikel erfolgen kann. Dieses ist eine Vorstufe des eigentlichen Aktivierungsprozesses, bei dem eine weitere Neuorientierung der submikroskopischen Elemente im Kortex stattfindet. Wahrscheinlich treten dann neue Haftpunkte im Netzwerk der Plasmamembran auf, wobei beim vollaktivierten Ei eine feinere Verteilung der stabförmigen Molekülgruppen vorliegt als beim Unbefruchteten (7).

Dass andererseits die Behandlung mit Bestandteilen, wie sie im „Fucoidin" enthalten sind, eine Wirkung auf die Oberfläche der unbefruchteten Eier hervorruft, zeigte sich an ihrer verminderten Streckung im Zentrifugenmikroskop. Entsprechende Nachbehandlung mit Perjodat wirkte dem entgegen.

Eine Ergänzung zu den vorliegenden Befunden liefern Resultate aus Versuchen, bei denen die Eier nach Behandlung mit Aminosäuren oder Proteinen zentrifugiert wurden (12). In Parallelversuchen wurde das Verhalten bei Hypertoniebehandlung verfolgt, wobei die auftretende Faltigkeit der Oberfläche und die zeitliche Dauer dieses Zustandes mit einer Kontrolle verglichen wurde. Die Wirkung bestimmter Aminosäuren und

Proteine ist u. A. darin zu sehen, dass sie die Zytoplasmarigidität mehr oder weniger herabsetzen und den Widerstand der Eier gegen die Verlängerung beim Zentrifugieren vermindern. Gleichzeitig bewirken sie eine Beschleunigung der Abrundung der Eier in hypertonischer Seewasserlösung im Anschluss an die zuerst auftretende Faltigkeit der Oberfläche. In diesem Zusammenhange sei erwähnt, dass unreife Eier durch ein längeres Andauern des Zustands der Faltigkeit bei Hypertoniebehandlung gekennzeichnet sind, dagegen wird bei reifen Eiern diese durch eine schneller auftretende Abrundung abgelöst (6, 11).

Es hat sich weiterhin gezeigt, dass Substanzen (12) die eine Herabsetzung der Rigidität des Kortex verursachen, gleichzeitig auch mehr oder minder befruchtungsverbessernde oder auch aktivierende Wirkungen haben. Sie schaffen dadurch offenbar die Voraussetzungen für die Vorgänge, die sich schrittweise nach dem Eindringen der Spermien abspielen. Auch für die erste Anheftung der Spermien kann der Zustand der Oberfläche von wesentlicher Bedeutung sein. Im Ei und in den Spermien lassen sich Faktoren nachweisen, die sich wechselseitig beeinflussen und damit Abschnittsweise die einzelnen Voraussetzungen für den Ablauf der Befruchtung schaffen. Eine Reihe von Versuchen (4) ergaben, dass sowohl Ei als auch Spermextrakte von Einfluss auf die Bildung der Befruchtungsmembran sind. Aus fast allen erwähnten Versuchen ergibt sich, dass die Herabsetzung der Rigidität des Kortex durch die erwähnten Verbindungen wichtige strukturelle Änderungen der Oberfläche verursacht, die primär bei der Aktivierung und Befruchtung der Eier von wesentlicher Bedeutung sind.

In Bezug auf die Zytoplasmarigidität zeigten mit Perjodat vollaktivierte Eier im Verlauf der ersten 15 Minuten nach der Ausbildung der Befruchtungsmembran die gleichen periodischen Änderungen wie normal Befruchtete. Eingehendere Untersuchungen über den weiteren Verlauf sind nicht durchgeführt worden. Zwischen nichtaktivierten und mit Perjodat präaktivierten Eiern ergaben sich im Zentrifugierversuch keine wesentlichen Unterschiede im Verhalten des Zytoplasmas.

Die Behandlung mit „Fucoidin“ erhöhte gleichzeitig auch die Rigidität des Zytoplasmas. Vermutlich trägt dieser Umstand mit dazu bei, dass sich der Widerstand der Eier gegen die Streckung beim Zentrifugieren verstärkt.

ZUSAMMENFASSUNG DER ERGEBNISSE

1. Die Wirkung von Natriumperjodat auf die Plasmamembran unbefruchteter Eier von *Psammechinus miliaris* wird mit Hilfe des Zentrifugenmikro-

skops studiert, wobei unter Anwendung einer konstanten Zentrifugalkraft die Streckung der Eier gemessen wird.

2. Durch Perjodat vollaktivierte Eier verhalten sich in bezug auf die Streckung so wie normal Befruchtete. Präaktivierte Eier zeigen eine ausgeprägte Verlängerung während des Zentrifugierens im Vergleich mit Nichtaktivierten.

3. Behandlung mit ,,Fucoidin" bewirkt eine Verminderung der Streckung im Vergleich mit unbehandelten Eiern. Der im ,,Fucoidin" enthaltene Bestandteil, der die Rigidität des Kortex herabsetzt und die Befruchtungsfähigkeit hemmt, wird als ,,Hemmungsfaktor F" bezeichnet. Anschliessende Perjodatbehandlung wirkt dem entgegen.

4. Nach Überführung in hypertonisches Seewasser ergaben sich unterschiedliche Plasmolysetypen bei den verschieden behandelten Eiern.

5. Im Zusammenhang mit den Befunden dieser Arbeit werden die Zustandänderungen der Plasmamembran und die Wirkungen von Perjodat und ,,Fucoidin" besprochen.

Am Ende dieser Arbeit ist es mir eine angenehme Pflicht, Herrn Prof. John Runnström für seine Anregungen zu den vorliegenden Untersuchungen und seine Hilfe meinen allerherzlichsten Dank auszusprechen. Weiterhin danke ich Herrn Prof. Nils Holmgren und Herrn Dr Gunnar Gustafsson von der Zoologischen Station, Kristineberg, für ihre freundliche Hilfe und die Beschaffung des für die Arbeiten benötigten Materials, sowie Herrn Dr Erik Vasseur vom hiesigen Institut für die Überlassung des in den vorliegenden Untersuchungen benutzten Präparats ,,Fucoidin".

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LETHAL AND MUTAGENIC EFFECTS OF ULTRAVIOLET RADIATION ON *GLOMERELLA* CONIDIA

C. L. MARKERT

Department of Zoology, University of Michigan, Ann Arbor, Michigan, U.S.A.

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In comparative studies of the mutagenic action of ionizing and ultraviolet radiation on conidia of the fungus *Glomerella* (5) it was evident that the shape of the mutation curve was different for the two types of radiation. The dose-mutation curve for the surviving spores was essentially linear for the ionizing radiations, but with the ultraviolet radiation the mutation curve rose with increasing dose to a peak and then declined with further increases in dose. Studies on the mutagenic effects of radiation on other fungi have generally yielded the same types of curves (1, 2, 3, 7). Although various supplementary treatments of the radiated spores have been shown to alter the frequency of mutants found at any dose, the basic shape of the curve is not changed by these treatments (10, 11).

The types of survival curves obtained by plotting the logarithm of the survivors against dose of radiation also vary with the type of radiation. Heavily ionizing radiation results in an exponential survival curve (8, 13) while the less densely ionizing x-radiation results in a sigmoid curve; many investigators have, however, obtained exponential survival curves with x-rays. The non-ionizing ultraviolet radiation has generally resulted in sigmoid survival curves (1), although Norman (6) obtained exponential survival curves with *Neurospora* microconidia after ultraviolet radiation. Ultraviolet radiation of bacteria has yielded either sigmoid or exponential survival curves depending upon the genetic makeup of the bacteria (12).

Of these various radiobiological effects perhaps the most perplexing has been the shape of the mutation curve with ultraviolet. No satisfactory explanation has yet been advanced for the decline in the mutation rate at high doses of ultraviolet (3). The present investigation was undertaken primarily to elucidate the factors involved in this peculiarity of the dose-mutation curve with ultraviolet and to relate this effect if possible to some characteristic of the survival curve.

MATERIALS AND METHODS

Conidia of the standard type (A^1B^1) of the fungus *Glomerella cingulata* were used in this investigation. The standard type differs by gene changes at two loci from the wild type (A^+B^+) which is usually the type isolated from nature. The A^1B^1 type produces no perithecia but when growing on appropriate media abundantly produces uninucleate haploid conidia dispersed over the mycelium (cf. Markert (5) for culture methods, etc.).

In the preparation of spore suspensions for irradiation the conidia were washed from the surface of month old cultures and filtered through cotton to remove any clumps of spores. The spores were then examined microscopically and the approximate concentration determined by counting with the aid of a hemocytometer. The density of the spore suspension was then adjusted to approximately one million per ml. Fifteen ml of this suspension were then added to an open Petri dish placed on a slightly inclined rotating turntable beneath a Westinghouse sterilamp which emits over 80 per cent of its radiant energy in the region of 2537 Å. The distance from the spore suspension to the bottom of the ultraviolet lamp varied between 9 and 9.5 cm. At this distance the intensity of the radiation was approximately 4 770 ergs/cm^2/sec. The depth of the spore suspension in the Petri dish averaged about 2.4 mm. A *Glomerella* conidial suspension of two million/ml absorbs less than 3 per cent of the

TABLE I

Response of sensitive and resistant *Glomerella* conidia to ultraviolet radiation.

Radiation dose in seconds[1]	Survivors per million	log survivors	number of colonies evaluated	Mutants	Per cent mutants	Data corrected to exclude resistant spores		
						colonies	mutants	Per cent mutants
0	1 000 000	6.0000	500	0	0	500	0	0
30	620 500	5.7927	310	21	6.8	310	21	6.8
60	244 600	5.3885	629	122	19.4	628	122	19.4
90	38 500	4.5855	642	233	36.3	637	233	36.5
120	6 550	3.8162	624	256	41.0	595	254	42.7
180	345	2.5378	717	183	26.0	224	124	55.4
240	194	2.2878	498	79	15.9	—	—	—
300	153	2.1847	101	17	16.8	—	—	—
360	134	2.1271						
500	51	1.7076						
600	22	1.3424						
900	20	1.3010						
1200	13	1.1139						
1500	8	0.9031						

[1] 4770 ergs/cm^2/sec.

incident radiation at 2537 Å with a path length of one cm as determined by measurements in a Beckman spectrophotometer. This method of irradiating the spores should insure that all spores are equally irradiated. As a check, however, several radiation experiments were performed using a rotating quartz flask to hold the spore suspension. The results from these experiments were not significantly different from those in which Petri dish containers were used.

During the course of the radiation 1 ml aliquots were removed at intervals and plated out at appropriate dilutions in a complete agar medium. In order to minimize photoreactivation all manipulations of irradiated conidia were performed in dim light and the Petri dishes containing the plated out spores were incubated in the dark at 25° C.

The control concentration of viable spores was determined by calculation from the dilution factor and the average number of colonies per Petri dish. The fraction of surviving spores at each dose of radiation was calculated in the same fashion and recorded as the number of spores surviving per million viable spores in the initial suspension.

In order to determine the mutation rate it was necessary to transfer all the viable colonies surviving on any selected Petri dish to agar slants in culture tubes. At four days and at six days of growth on the agar slants at 25° C an evaluation of all the colonies was made. Those which were visibly different from the control cultures at either time were classified as mutant (5). The percentage of mutant colonies among the survivors was then plotted against dose to obtain the mutation curve.

RESULTS

The results of these experiments are summarized in Table I and Fig. 1. In each of eleven separate experiments on the lethal effects of ultraviolet radiation on *Glomerella* conidia a plot of the log of the surviving spores against dose gave a distinctly sigmoid curve which leveled off to form a long plateau. The individual values (survival curve) plotted in Fig. 1 represent the average of these eleven experiments.

The points on the mutation curve represent the percentage of mutants found among the viable colonies at each dose of radiation. The data on mutation are listed in Table I. The shape of the mutation curve corresponds with that found after ultraviolet radiation of other fungus spores (10, 1). The curve first rises to a peak and then declines with increasing dose of radiation.

The sigmoid survival curve clearly indicates a cumulative effect of the radiation and on the assumption of a multiple hit target hypothesis would correspond to about a 7 hit curve. This type of survival curve would also be obtained as the consequence of an indirect effect of the radiation producing a toxic substance with a threshold concentration for killing the spores.

The long plateau at the end of the survival curve implies that the irradiated spore suspension is not homogeneous but is composed of spores with different degrees of sensitivity to the ultraviolet radiation. The shape of the curve does not critically indicate whether the heterogeneity was present at the outset or was induced by the radiation itself. However, the height of the plateau above the base line—that is, the fraction of resistant spores in the suspension, varied with the age of the culture from which the spore suspension was obtained, the older cultures containing more of the resistant spores. This correlation between the number of resistant spores and the age of the spore culture leads to the conclusion that the *Glomerella* spore suspensions were heterogeneous before radiation. In order to insure identical responses to radiation, it was necessary to use spore cultures of exactly the same age and environmental history.

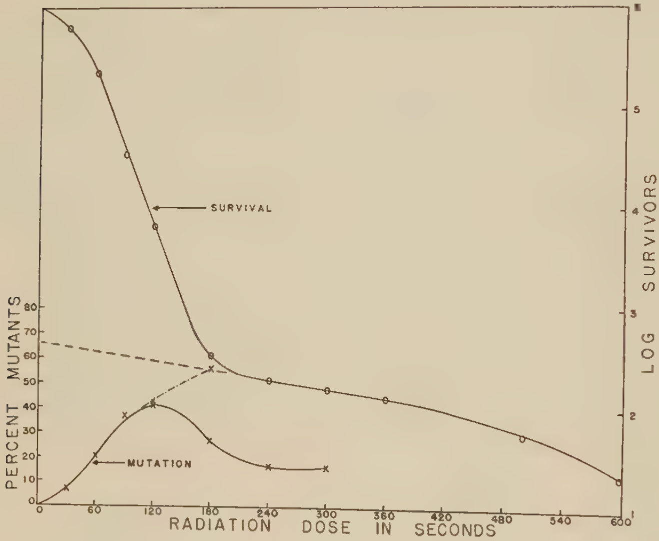

Fig. 1. Relationship of survival and mutation to dose of ultraviolet. The higher points for mutation at 120 and 180 seconds are for sensitive spores alone.

Several experiments were performed in an effort to identify and characterize the resistant fraction of the spore population. Since a genetic basis for radiation resistance has been demonstrated in *E. coli* (12), the possibility that the resistant spores were mutants was tested. Colonies were grown from resistant spores and allowed to sporulate. The conidia were then subjected to the same radiation treatment given the conidia from the stock cultures. The survival curves describing the response of these conidia from the resistant colonies were not significantly different from the survival curves obtained with the stock conidia. These tests also exclude the possibility that the resistant spores might owe their resistance to a temporary diploidy produced by a precocious chromosomal division. Such a diploid spore on germination might yield two individually inviable haploid colonies which could survive, however, in symbiotic partnership. However, after sporulation such a mixed colony would be detected by the inviability of its spores. Thus the resistant spores must represent physiological or morphological variants without any genetic basis.

Although the technique of preparing the spore suspension should exclude clumps of spores, the possibility was examined that a few clumps were formed during the radiation and that these clumps provided the necessary protection to the centrally located spores to enable them to survive high doses of radiation. A microscopic examination was therefore made of the Petri dish cultures of young colonies growing from spores exposed to very high doses of radiation (1500 seconds). This examination failed to reveal any clumps of spores but it was immediately apparent that in every instance the germinating spores were highly pigmented. Normally *Glomerella* conidia appear pink in large masses but essentially colorless when viewed individually under the microscope. The pigmented resistant spores were black as shown in Fig. 2. The black pigment is presumably a melanin produced as the result of tyrosinase activity. *Glomerella* conidia as well as the mycelia have been shown to contain large quantities of tyrosinase (9). No explanation is at present available to account for the fact that only a very small fraction of the spore population becomes pigmented although all of the spores contain the necessary enzyme for synthesizing the melanin.

The presence of resistant pigmented spores in a normal spore population suggested an explanation for the shape of the mutation curve (Fig. 1). At increasing doses of radiation the surviving spores would be composed of increasing proportions of the resistant variety (resistant both to the mutagenic and lethal action of the ultraviolet) and thus the proportion of mutable spores (and of mutants) would decline at increasing doses. By extrapolating the plateau portion of the survival curve back to zero dose an estimate of the number of resistant spores per million spores of the initial population may be obtained. In spore cultures of the age (one month) used in these experiments the resistant spores number about 470 per million spores.

The mutation frequencies used in plotting the mutation curve (Fig. 1) were based almost entirely upon mutations of sensitive spores. In order to describe accurately the frequency of mutation among sensitive spores it is necessary to make a correction for the presence of resistant spores in the initial spore population. The resistant spores surviving at any given dose may be estimated from the appropriate point on the extrapolated portion of the plateau of the survival curve (dashed line in Fig. 1). Although the extrapolated portion of the curve should be sigmoid rather than linear as drawn, the deviation from linearity at doses of 120 and 180 seconds would be too small to affect the data significantly. After 240 seconds of radiation essentially only resistant spores survive. The low percentage of mutation at this dose indicates that even the resistant spores are eventually mutated or killed by the ultraviolet radiation.

Thus in calculating the mutation rate for sensitive spores alone it is necessary to subtract the surviving resistant spores from the number of colonies evaluated and also to subtract the mutants contributed by the resistant colonies from the total mutants found. The mutants from resistant colonies were calculated on the assumption of a linear dose-mutation relation for these resistant spores up to 240 seconds at which point the mutation rate was observed to be approximately 16 per cent. The corrected mutation frequencies for sensitive spores are listed in Table I and are depicted by the dotted mutation curve in Fig. 1. It is apparent that the mutation rate for sensitive spores alone does not decline with increasing doses of radiation.

The existence of melanin pigment in a few of the spores from month old cultures serves to separate the spore population into two distinct and fairly homogeneous groups each with its own distinct sensitivity both to the mutagenic and to the lethal effects of the ultraviolet radiation. The relationship between the mutagenic and lethal effects of the radiation is shown by the fact that the ratio of the slopes of the mutation curves (about 6:1) for the two types of spores corresponds closely to the ratio of their relative resistance to the lethal effects of the radiation.

DISCUSSION

The survival and mutation curves obtained in this investigation with spores of *Glomerella* are very similar to those obtained by Hollaender and Emmons (1) with ultraviolet irradiated spores of the fungus *Trichophyton mentagrophytes*. These investigators and others (10) have generally attributed the decline in the mutation rate at high doses of ultraviolet to an increased sensitivity of mutated spores to the lethal action of the radiation. Although such an hypothesis is consistent with the previously known facts, it would appear from the *Glomerella* example that a more probable explanation must be based upon the presence of resistance-conferring morphological or physiological characterstics in a small fraction of the spores in the initial population.

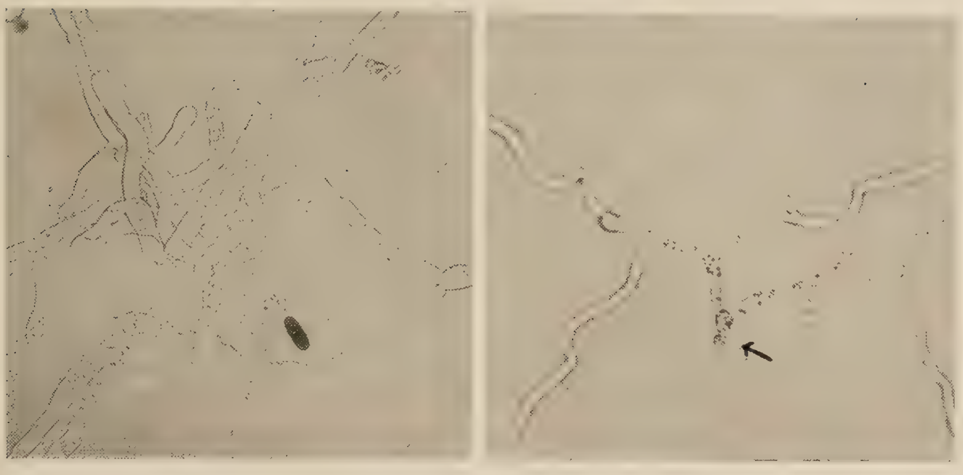

Fig. 2. (Left) Resistant pigmented spore germinating after exposure to 1500 secs of ultraviolet radiation. (Right) Unirradiated colorless sensitive spore germinating.

So far as susceptibility of mutants to ultraviolet is concerned there is direct evidence that numerous mutants of *Glomerella* do not differ from standard type in their response to ultraviolet. In experiments on ultraviolet induced back mutations several nutritional and morphological mutants were exposed to ultraviolet at varying doses. Their survival curves were not significantly different from those of the standard type. Unfortunately these experiments were done before the melanin basis for ultraviolet resistance was known and no effort was made to pick mutants with greatly different amounts of tyrosinase. Mutants differing in tyrosinase content might well show corresponding variations in the proportion of resistant pigmented spores produced in a spore culture.

In quantitative irradiation experiments with the fungus *Neurospora crassa,* Hollaender *et al.* (3) also found the typical rise and decline of the mutation curve with increasing doses of ultraviolet. They suggested that the initial spore suspension might be heterogeneous in respect to the amount of the radiation absorbed by each spore because of variations in the ultraviolet absorption by the spore wall and cytoplasm at different ages of development. Efforts by them to test this hypothesis on spores of different ages yielded inconclusive results. Although this hypothesis was not clearly verified for *Neurospora* conidia, it is evidently the correct explanation for the behavior of *Glomerella* conidia, in which protective melanization is partly a function of age. Since *Neurospora* mycelia contain tyrosinase (4) it may yet be found that the conidia also contain tyrosinase and variable amounts of melanin pigment.

In another organism the B strain of *E. coli*—Witkin (12) has shown that the survival curve after ultraviolet irradiation decreases in slope at high doses. Although genetically resistant bacteria occur among large populations of this strain, these genetically resistant bacteria are not numerous enough to account for the change in slope of the survival curve. Some non-inherited protective characteristic must be present in part of the bacterial population.

The fact that melanin pigment absorbs both visible and ultraviolet light is a fortuitous combination of characteristics which makes the resistance-conferring character visible under the microscope. However, there may well be other types of invisible chemical or physical changes in fungus spores which confer varying amounts of resistance to ultraviolet radiation. The melanized *Glomerella* conidia are only one example that such mechanisms do exist.

SUMMARY

1) The survival curves of ultraviolet irradiated *Glomerella* conidia have a sigmoid shape. The sigmoid survival curve may be described as a 7 hit curve (approximately) or as indicating radiation-produced toxic substances with a threshold for lethal action.

2) At high doses of radiation the survival curve levels off to form a long plateau. This plateau in the curve indicates the presence of a small proportion of resistant spores among the sensitive population. The resistant spores may be observed under the microscope and are characterized by the presence of melanin pigment in the spore in contrast to the great majority of *Glomerella* conidia which appear colorless. The proportion of resistant spores increases with increasing age of the spore culture.

3) With increasing dose of ultraviolet radiation the mutation curve rises to a peak and then declines at higher doses of radiation. This decline in the frequency of mutants among the surviving spores is due to an increasing proportion of resistant pigmented spores among the surviving population at high doses. The mutation curve for sensitive spores alone does not decline at the higher doses of radiation.

The author wishes to acknowledge the careful assistance of Miss Bonnie Gobble and Miss Patricia A. Caughey. This investigation was aided by grants from the Atomic Energy Commission under contract No. AT (11-1) –70 project No. 4 with the University of Michigan.

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AN EXPERIMENTAL STUDY OF THE BIOSYNTHESIS OF THE RESERVE GLOBULINS IN PEA SEEDS

O. SNELLMAN and C. E. DANIELSSON

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

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Our present knowledge about protein biosynthesis is very limited because of the relatively great difficulty in finding a natural system simple enough to permit a reliable quantitative study of the process. Earlier investigations carried out at this Institute on ripening pea seeds showed that this system is convenient for the study of protein biosynthetis (1, 2).

MATERIALS AND METHODS

From seeds of *Pisum sativum* two well-defined globulin components, vicilin and legumin, which were shown to have different amino acid compositions (4), were isolated according to the following method. Pea seeds were gathered at eight different stages of ripening with four days between every stage. The seeds of each stage were separated from the seed coats. Some of the seeds, about 50 per cent of the total of each grade, were preserved by freezing to —20° C and the remainder was stored at room temperature. After grinding, extractions with 0.2 M NaCl, buffered to pH 7 with phosphate, were carried out on the two lots obtained for each stage of ripening. These extracts were dialyzed against the extraction buffer. In this way any nitrogeneous product of low molecular weight was removed from the extract. The amounts of these products were assayed quantitatively by Kjeldahl nitrogen determinations before and after the dialyses. The extracts, now containing the globulins (vicilin and legumin) and the albumins, were then dialysed against water, by which procedure the globulins were precipitated. The albumins remained in the supernatant and were quantitatively assayed by nitrogen determination. The precipitate, containing the globulins, was dissolved in 0.2 M NaCl, pH 7, and the total amount of globulin assayed by nitrogen determination. The ratio between the amounts of vicilin and legumin was obtained by analysis of the solution in the ultracentrifuge. Amino nitrogen determinations were carried out on the extracts, using acetone titration on a micro scale according to Linderstrøm-Lang (7).

With the methods described above, the changes in the amounts of globulin nitrogen (vicilin and legumin separately), albumin nitrogen, dialysable nitrogen and amino nitrogen could be evaluated at different stages of the ripening process. The values already published in the papers cited above have been used in this communication for a more detailed interpretation of the biosynthesis of the reserve proteins in ripening pea seeds.

RESULTS

The mobilization of the low-molecular weight nitrogeneous products. Table I and Fig. 1 give the experimental values obtained for the amount of total globulin nitrogen (i.e. vicilin + legumin) and dialysable nitrogen at different stages of the ripening process. The amount of globulin nitrogen in ripe seeds was extrapolated from the experimental curve, and the value obtained was taken as 100 per cent. All other globulin values were calculated as percentage of this value. The decrease in dialysable nitrogen was recalculated in the same way. The excellent agreement between the decrease in dialysable nitrogen and the increase in globulin nitrogen clearly suggests that the reserve proteins are quantitatively synthesized from dialysable nitrogenous products.

Fig. 2 shows the amounts relative to those in ripe seeds of vicilin and legumin at different stages of the ripening process, obtained from the values of globulin nitrogen in Table I, and the from ratios $\frac{\text{vicilin}}{\text{legumin}}$, as determined by ultracentrifugation. From the curves in Fig. 2 it can be seen that vicilin and legumin are synthesized by a zero order process with different velocity constants, vicilin being more rapidly synthesized than the legumin of higher molecular weight. This may be interpreted to be due to the fact that the active surfaces of the synthesizing enzymes are saturated with substrate and, therefore, that the rate determining process is the desorption of the products from the active surface of the enzymes.

Table II and Fig. 3 show that high amino nitrogen values are obtained in very young seeds. These high values are mainly due to free amino acids. The polypeptides and the globulins will only give a small contribution as can be seen from the low values obtained at the end of the ripening process. The decrease in amino nitrogen in the beginning of the ripening process is essentially a measure of peptide synthesis. Fig. 4 was obtained in the following way. The values of the amounts of globulin nitrogen and amino nitrogen were taken from the smoothed curves in Fig. 1 and Fig. 3 respectively. In Fig. 4 the values of amino nitrogen were used directly from Fig. 3, but instead of using the globulin values (x per cent) from Fig. 1 directly, (100-x per cent), its arithmetic complement was used. It is quite clear from the discrepancies between the two reaction curves (Fig. 4) that the synthesis goes through different steps and that the reaction in the step to oligopeptides is more rapid than in that to polypeptides.

Fig. 4 shows that during the most rapid synthesis of the globulin the decrease in amino nitrogen is retarded. This indicates that during this step in the synthesis, i.e. from oligopeptides to polypeptides, amino nitrogen is liberated.

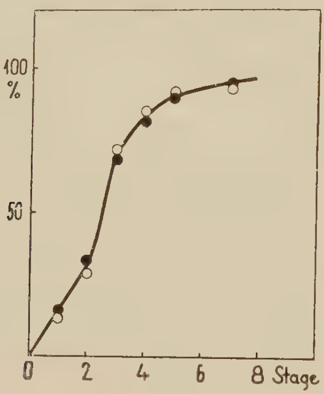


Fig. 1.

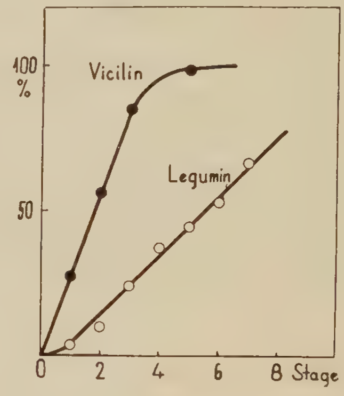


Fig. 2.

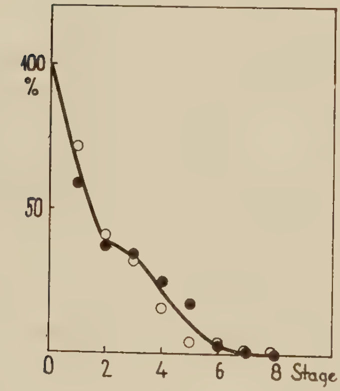


Fig. 3.

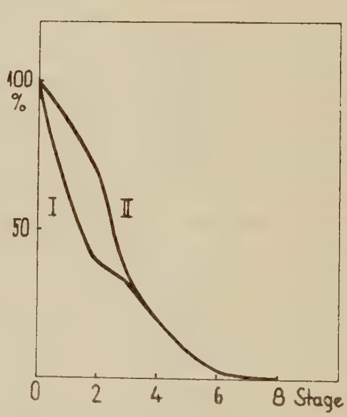


Fig. 4.

Fig. 1. The amount of globulin in percentage of the final amount in ripe seeds at different stages of the ripening process (solid circles). The decrease in dialysable nitrogen, calculated in percentage of the total decrease during the whole ripening process (open circles). The abscissa represents different stages of the ripening process (four days between every stage).

Fig. 2. The amount of vicilin and legumin in percentage of the final amount at different stages of the ripening process.

Fig. 3. The decrease in amino nitrogen in pea seeds, dried at room temperature (open circles) and freeze-dried (solid circles) at different stages of the ripening process, expressed as percentage of the total decrease during the whole ripening process.

Fig. 4. Smoothed curves showing the changes in amino nitrogen (NH_2-N) and globulin nitrogen at different stages of the ripening process. Amino nitrogen is expressed in percentage of the total change. The same is true of globulin nitrogen, but instead of plotting the actual value x per cent, (100-x) per cent was used.

TABLE I

Nitrogen distribution in NaCl extracts from pea seeds at different stages of the ripening process. In one experiment, 1.50 g of dried seed material were extracted. Globulin nitrogen was determined as nondialysable N — albumin N.

Stage	Total N	Dialysable N				Globulin N		
	mg	mg	per cent of total N	Normalized 0 to 100 per cent	Decrease per cent	mg	per cent of total N	Normalized 0 to 100 per cent
0			[1]69.0	100	0		[1]0	0
1	69.7	44.2	63.4	87.2	12.8	4.7	6.8	16.2
2	69.3	39.2	56.6	71.8	28.2	10.0	14.4	34.3
3	61.5	22.9	37.2	27.7	72.3	18	28.6	68.0
4	62.8	19.9	31.7	15.2	84.2	21.5	34.2	81.4
5	55.0	15.7	28.5	8.0	92.0	20.6	37.5	89.2
6	57.9	15.5	26.8	9.1	95.9	17.8	32.5	77.4
7	53.2	15.0	28.2	7.3	92.7	20.8	39.2	93.6
Ripe seeds			[1]25.0	0	100		[1]42.0	100

[1] denotes extrapolated values.

TABLE II

Amino nitrogen content (g-equivalents/ml × 10^5) in water extracts of pea seeds at different stages of ripening.

Stage	Seeds preserved by freezing		Seeds dried at room temperature	
	g-equivalents/ml × 10^5	Normalized 100 to 0 per cent	g-equivalents/ml × 10^5	Normalized 100 to 0 per cent
0.		[2]100		[2]100
1.	9.35	54.0	10.58	60.0
2.	7.16	33.4	7.56	34.4
3.	6.95	31.4	6.66	26.8
4.	6.00	22.3	5.04	13.1
5.	5.27	15.3	3.91	3.5
6.	3.73	6.7	3.75	2.1
7.	3.66	0.0	3.41	—
8.	3.69	2.9	3.52	1.7

[2] represents extrapolated values

DISCUSSION

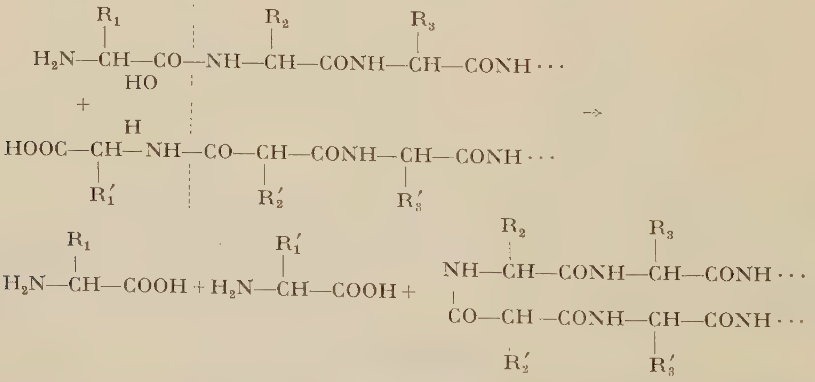
A possible basic mechanism of protein biosynthesis. We will now try to give a picture of the basic mechanism of the synthesis of the reserve proteins.

Schulze and Winterstein (9) found different proportions of asparagine and arginine in the pods and in the unripe seeds indicating a redistribution of the amino acids before they build up proteins. These findings are being reinvestigated at this Institute in the light of modern methods. Accepting the gross features of Schulze and Winterstein's findings one is led to assume that the first step in the synthesis is an alteration in the distribution of the amino acids so that we have a distribution which is necessary for the subsequent steps in the synthesis of a certain protein.

The next step in the process is a building up of oligopeptides by condensation of amino acids. This is a relatively rapid process as to indicate from the curve obtained by combining the globulin curve and the curve for the disappearance of the amino nitrogen. Oligopeptides containing about 2–6 amino acids have been isolated (3) and further experiments are in progress. This step in the synthesis must be done with expenditure of much energy. It is therefore to be assumed that energy rich phosphate bonds here play a role (5).

The curve showing the synthesis of the total globulins is an expression for the formation of non-dialysable polypeptides from oligopeptides. The formation of oligopeptides from amino acids is a more rapid process. During the time when the globulin synthesis is most pronounced there is a retardation in the disappearance of the amino nitrogen which indicates a liberation of amino nitrogen during the synthesis of polypeptides (Fig. 4).

The simplest way to explain this stage is to assume an enzyme or enzyme complex which rearranges the amino acid moieties of the oligopeptides according to the following equation:



$$\begin{array}{l}
H_2N\text{—}\overset{R_1}{\overset{|}{CH}}\text{—}\underset{HO}{CO}\text{—}NH\text{—}\overset{R_2}{\overset{|}{CH}}\text{—}CONH\text{—}\overset{R_3}{\overset{|}{CH}}\text{—}CONH\cdots \\
+ \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \rightarrow \\
HOOC\text{—}\underset{R'_1}{\underset{|}{CH}}\text{—}\overset{H}{NH}\text{—}CO\text{—}\underset{R'_2}{\underset{|}{CH}}\text{—}CONH\text{—}\underset{R'_3}{\underset{|}{CH}}\text{—}CONH\cdots
\end{array}$$

$$H_2N\text{—}\overset{R_1}{\overset{|}{CH}}\text{—}COOH + H_2N\text{—}\overset{R'_1}{\overset{|}{CH}}\text{—}COOH + \begin{array}{l} NH\text{—}\overset{R_2}{\overset{|}{CH}}\text{—}CONH\text{—}\overset{R_3}{\overset{|}{CH}}\text{—}CONH\cdots \\ | \\ CO\text{—}\underset{R'_2}{\underset{|}{CH}}\text{—}CONH\text{—}\underset{R'_3}{\underset{|}{CH}}\text{—}CONH\cdots \end{array}$$

Such an equation has not been proposed earlier and it is interesting to compare it with that proposed by Linderstrøm-Lang (8).

In our equation one amino nitrogen group is gained at every reaction of two oligopeptides. It is thus possible to explain the difference between the curves of the amino nitrogen and the protein nitrogen. Furthermore the energy for the synthesis of the new bond is derived from the scission of the two amino acid residues. The energy principle in this instance is analogous to that involved in the synthesis of a dextran molecule from sucrose by the enzyme sucrase (6). There the reaction occurs by the utilization of the energy released in the hydrolysis of the gluco-fructose bond. The mechanism given here also offers possibilities for explaining how cyclo-peptides, which occur in certain proteins, are formed.

The high molecular weight peptides formed at this stage can be seen in the ultracentrifuge using freeze-dried material. One of us (1) has observed that during the first stages of the ripening process polydisperse high molecular weight peptides or pre-globulins predominate among the materials of high molecular weight. In the later stages of the ripening processes this material cannot be seen, probably because the small amounts then present are hidden in the diagrams by the large amounts of globulins.

Ripening pea seeds appear to offer a system in which the protein synthesis can be followed throughout its different stages. In general little information can, however, be obtained about the last step from high molecular weight polypeptide to globulin. This step seems to proceed rapidly.

It seems that neither the long ranging forces of Rothen nor any single process picture is necessary to explain protein biosynthesis in pea seeds. Such views do not allow for the occurrence of steps in such synthese.

A certain genetically fixed distribution of the amino acids may perhaps explain the formation of oligopeptides with certain definite sequences.

This distribution also allows for the common character of the proteins. In this connection it is noteworthy that many globulins have the same basic structure of amino acids.

In the last steps of these syntheses nucleic acids may be involved, but exactly what their role is in these processes cannot be ascertained from the experiments which have been done up to now.

SUMMARY

1. Quantitative analyses for different categories of nitrogeneous substances were carried out on pea seeds at different stages of the ripening process.

2. Well-defined globulins are quantitatively synthesized from dialysable nitrogeneous products.

3. The synthesis of the well-defined globulins results from several steps.
4. A new reaction mechanism for the formation of long peptide chains from short ones is discussed.

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MESSUNGEN DES SILBER-DICHROISMUS AN AMYLOID-KOMPLEXEN *IN VITRO*

H. H. PFEIFFER

Laboratorium für Polarisationsmikroskopie, Bremen, Deutschland

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Unter den Methoden zur Analyse des leptonischen Baues der Zelle wird die Polarisationsoptik, wenn sie auch als indirektes Verfahren zum Unterschied von der modernen Elektronenmikroskopie nicht-auflösbare Zellstrukturen nicht direkt sichtbar machen kann, ihren bevorzugten Platz behalten müssen, weil ihr die *lebende* Zelle zugängig ist, die sich andern einschlägigen Verfahren meist versagt, alle Ergebnisse an fixierten Objekten aber gründlicher Kontrolle am lebenden Objekt bedürfen [15]. Erst recht aber gilt der postulierte Wert aller polarisationsoptischen Methoden, sobald ein *quantitativer Aspekt* wie zu fordern berücksichtigt wird [8, 9, 13, 4]. Deswegen sind in Ergänzung und Erweiterung letztjähriger Messungen des Congorot-Dichroismus amyloidisch veränderter Gewebselemente *in vitro* [11] neuerdings Bestimmungen des *Silber-Dichroismus* an gleichfalls nach der Technik von A. Paravicini in Kaninchen-Heparinplasma und Gewebeextrakt ungeteilt ausgepflanzten Explantaten mit und ohne amyloide Stoffwechselstörungen vorgenommen worden. Über die hauptsächlichen Ergebnisse soll hier unter gleichzeitiger Diskussion der Bedeutung der Methode kurz berichtet werden.

METHODISCHE VORBEMERKUNGEN

Für die *quantitative Bestimmung des Silber-Dichroismus* durch separate Ermittlung der beiden Absorptionskoeffizienten k_a, k_o ist eine als zweckmässig erachtete Methode angewandt worden, wie sie ähnlich Berek [2] mit bestem Erfolge ausgebildet hat. Hervorragend geeignet ist dafür das Leitzsche *Mikrophotometer* (Abb. 1) zur Messung der Lichtdurchlässigkeit. Unter Berücksichtigung des Verlusts durch Reflexion resultiert bei senkrechter Incidenz der Absorptionskoeffizient k exakt aus der Lichtdurchlässigkeit D, der durchstrahlten Dicke des Objekts d, seinem relativen Brechungskoeffizienten n gegenüber dem Medium, der Wellenlänge (im Vakuum) λ und dem Reflexionsvermögen R bei den üblichen Werten für π und e zu:

$$k = \frac{2\ \log(1-R) - \log\ D}{4\ \pi d \cdot \log\ e} \cdot \lambda_o$$

$$= \frac{2\ \log \dfrac{4}{(n+1)^2} - \log\ D}{4\ \pi d \cdot \log\ e} \cdot \lambda_o,$$

d. h. ausser der angewandten Wellenlänge des Lichts (für „weiss“ = 550 mμ) benötigen wir die Grössen d, n und D. Die Messung der *Präparatdicke* erfolgt mit reichlicher Genauigkeit nach der objektiven oder der subjektiven Newtonschen Interferenzmethode [6], die Bestimmung der *Durchlässigkeit* D mittels dem am Mikroskop angebrachten Mikrophotometer (Abb. 1) nach Berek [2] als Quadrat aus dem Quotienten der sin der abgelesenen Winkel (σ_0 bezw. σ) gleicher Feldhelligkeit ohne (σ_0) und mit (σ) Auflegen des präparierten Explantats auf den zur Nullstellung benutzten Objektträger aus Klarglas, und für die Ermittlung des *Brechungskoeffizienten* n sind mehrere Verfahren als brauchbar bekannt [12], am bequemsten vielleicht die Abschätzung der Brechungszahl aus dem Verhalten der F. Beckeschen Lichtlinie beim Einstechen von Glasnadeln einer geeigneten Kollektion Glasproben planvoll gesteigerter Refraktion [10]. Eine gewisse Sorgfalt und Genauigkeit dieser dreierlei Bestimmungen ist erwünscht, schon weil die Werte in der Grössenordnung der Wellenlängen in die Rechnung eingehen. Sobald in solcher Weise der ausserordentliche Absorptionskoeffizient k_a parallel zur Längserstreckung der Strukturen des Explantats und der ordentliche Koeffizient k_o parallel zur Quererstreckung separat gemessen worden sind, resultiert der gesuchte Dichroismus Δk als Differenz beider:

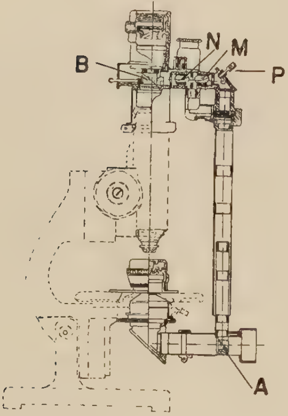

Abb. 1. *Schnitt durch Leitz' Mikrophotometer* nach M. Berek. Es bezeichnet A im Eintrittsrohr die Lenkung des Lichtes in zwei differente Wege durch das Mikroskop und den Tubus des Photometers; B ein ein- und ausschaltbares Photometer-Prisma (mit Irisblende vor der Eintrittsöffnung) zum Vergleichen der beiden Lichtanteile im Gesichtsfelde des Okulars; M, N den fixen und den unter Messkreis drehbaren Nicol zum Abschwächen des durch den Tubus des Photometers kommenden Lichtes; P Justierschrauben zur Zentrierung der beiden Austrittspupillen zueinander, welche bei Einschalten von B und bei Einstellen von N auf maximale Helligkeit sichtbar werden. Zur abschliessenden Justierung dient eine über das Okular zu stülpende Hilfslupe.

$$\Delta k = k_a - k_o.$$

Wenn $k_a > k_o$, liegt positiver (im grössten Teil des Spektrums), im Falle von $k_a < k_o$ dagegen negativer Δk (im kurzwelligen Teil des Spektrums) vor (Abb. 2).

Sowohl der Dichroismus Δk, als auch die Doppelbrechung Δn der versilberten Strukturen der Explantate sind weitgehend von dem *Gehalt eingeführten Silbers* abhängig. Daher ist in Anlehnung an E. Kolbes Jenenser Dissertation (1912) der beim Veraschen des versilberten Gewebes verbleibende Rest in HNO_3 gelöst und das Ag als Chlorid niedergeschlagen und bestimmt worden. Bei der *Imprägnationsmethode* nach Weber [1], auf welche die Wahl fiel, dürfte der Gehalt an Ag in den Explantaten zwischen 0,05 und 0,08 Volum-Prozent geschwankt haben.

ERGEBNISSE UND DISKUSSION

Durch die Webersche Imprägnation wird die *Gesamtdoppelbrechung* Δn der als (Stäbchen-) Mischkörper im Sinne O. Wieners bekannten Amyloide [11] herabgesetzt. Der Grund dafür lässt sich nicht sicher angeben. Die Δn-Kurve der Ag-imprägnierten Molekelketten ergibt in bezug auf verschiedene Wellenlängen den charakteristischen Verlauf durch ein Minimum. Die Grössenordnung der dabei gefundenen *Eigendoppelbrechung* liegt wie an Präparaten nach Congorot-Färbung bei 3,5 bis $7 \cdot 10^{-4}$ und die *Lichtrefraktion* amyloiden Zellmaterials bei $n = 1{,}556$ bis 1,561.

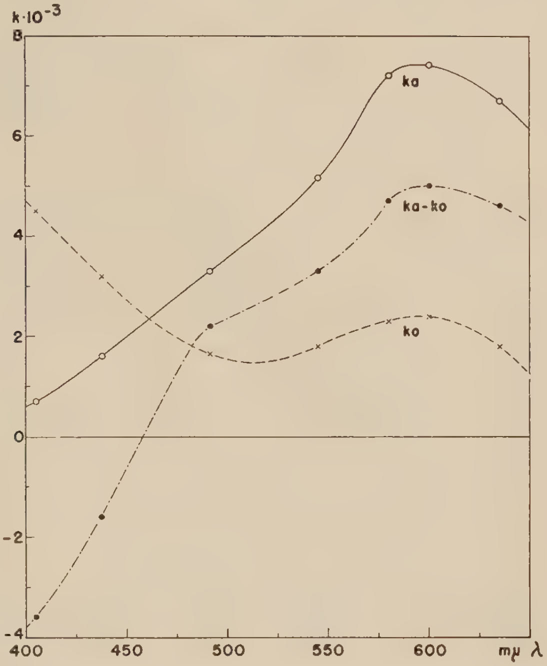

Abb. 2. Verlauf der *Kurven des ausserordentlichen und des ordentlichen Absorptionskoeffizienten* k_a, k_o und des daraus resultierenden *Dichroismus* $\Delta k = k_a - k_o$ durch das Spektrum des sichtbaren Lichts bei Silberimprägnierung amyloidisch veränderter Teile der Explantate.

Der gemessene *Ag-Dichroismus* (indigoblau–strohgelb) folgt in bezug auf die Wellenlängen einer fast ganz parallelen Kurve (Abb. 2 und 3). Wiederum wird die Babinet-Zochersche Regel (11) auch beim Ag-Dichroismus bestätigt, d. h. der stärker absorbierte Strahl wird im langwelligen Spektralbereich stärker, im kurzwelligen aber schwächer gebrochen. Berechnungen mit Hilfe der Wienerschen Absorptionsformeln zur Analyse des Metalldichroismus [3] sind bis soweit wegen erheblicher rechnerischer Schwierigkeiten noch zurückgestellt worden; man muss auch wohl fragen, ob das Objekt für die Aufgabe auch nur entfernt so brauchbar wie die von Frey-Wyssling und Wälchli [3] dazu herangezogenen Ramiefasern sein wird.

Aus dem Δk Ag-imprägnierter Amyloide muss auf das Vorkommen intermicellarer Räume und leptonischer Micelle beide parallel zu mikroskopischen Längsstrukturen der Objekte geschlossen werden. Die Deutung des Δk durch Metallimprägnation im Sinne von H. Ambronn und A. Frey-Wyssling als *gerichtete Einlagerung* geeigneter Leptonen des Metalls ist wegen gelegentlichen Ausbleibens des Δk trotz reichlicher Metallsalzaufnahme auch heute noch teilweise unsicher. Aus dem Vergleich der Messungen mit steigenden Ag-Anteilen (Abb. 3) wage ich fürs erste noch keine Folgerung zu ziehen. Es sieht aber so aus, als ob das Objekt zum Auftreten des Δk mit Ag-Salzen spezifische Eignungen aufweisen muss, welche unter Umständen auf *besonderer Molekelform* und damit ermöglichten Bindungen von Nebenvalenzen der Hydratgruppen des Substrats oder ähnlichen Faktoren beruhen mögen [7]. *Gegen* die erschlossene „Einlagerung" dichroitisch wirkender Ag-Leptonen sind keine Befunde verzeichnet worden. Wenn die Amyloide von G. Friedel als mesomorph angesprochenen Gebilden verwandt sein

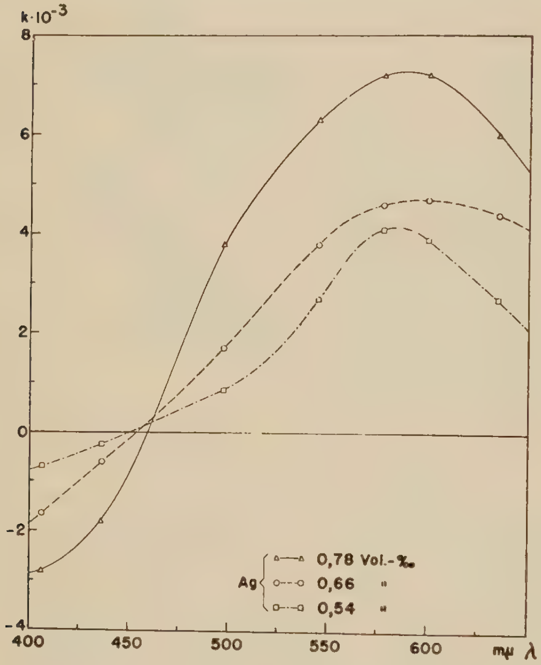

Abb. 3. *Einfluss unterschiedlichen Ag-Gehalts* der versilberten Amyloidkomplexe *auf den Silberdichroismus Δk bei* verschiedenen Wellenlängen (λ) des Spektrums sichtbaren Lichtes.

sollten, würde die erwartete Metall-Einlagerung bei der höheren Mischbarkeit bezw. Lösefähigkeit solcher Zwischenphasen noch an Wahrscheinlichkeit wachsen.

Differenzen zwischen stärkerem Δk des Amyloids und schwächerem anderer Anteile der Explantate sind gewiss mit der *dichteren Struktur* ersterer, einer ausgesprochenen Lockerstruktur des relativ gequollenen Explantatmaterials zu erklären. Das hat sich auch bei dem Einfluss des Quellungszustandes des Gewebes auf die Verzögerung der Reduktion an den Ag-Ionen bei Bielschosky-Versilberung gezeigt [14]. Damit verwandte Verhältnisse gelangen wohl bei Vorkommen von Störungen durch *Rheumatoide* [5] zur Ausbildung.

Die *Dispersion des Δk des Amyloids* und der übrigen Bestandteile des Explantats ist wie bei andern einigermassen farblosen Substanzen durchgängig normal. Da bei negativem Δk im kurzwelligen Bereich negative Δn im längerwelligen, aber positive Δn im kurzwelligen resultiert, muss sich durch Zusammenwirken von amyloidem Substrat und Ag-Salz im langwelligen Teil des Spektrums eine gegenseitige Verstärkung, im kurzwelligen Bereich eine Schwächung ergeben. Allerdings ist der Nachweis dieser Anomalie bei den höheren Interferenzfarben infolge Eigenfarbe des Ag-imprägnierten Präparats einigermassen heikel, gelingt aber ganz regelmässig bei schwacher Anisotropie, will sagen: in relativ dünner Präparatschicht.

Wie in andern Fällen hat sich die optische Anisotropie der Zelle wieder als dynamisches Phänomen erwiesen, zu dessen Analyse im Zellgeschehen die *polarisationsoptische Technik* mittels *quantitativer Ausgestaltung* zu einer physiologischen Angriffswaffe auf die in den Lockerstrukturen der Biogele wirksamen Kräfte entwickelt werden muss [8, 9, 13, 4]. Es kann aber nicht mehr speziell dargelegt werden, wie neben Doppelbrechung und Doppelabsorption (Dichroismus) auch Doppelbeugung (Beugungspolarisation) und überhaupt *alle Sparten optischer Anisotropie* durch das sichtbare Spektrum hindurch und darüber hinaus ebenso wie Röntgenographie und alle mechanisch-mikrurgischen, Quellungs- und andern Experimente vergleichend in den Dienst gestellt werden müssen, soll dem *Ziel der leptonischen Zellanalyse* nähergekommen werden [9].

I am greatly indebted to my friend Dr. G.-A. Johansson (Stockholms Sjukhem) for providing me with so many slices unstained and stained, and for his suggestions and helpful discussions during his visit. Also I owe a great debt of gratitude to Prof. A. Frey-Wyssling (Zürich) for his courtesy in providing me with a lot of belonging papers. Finally it is a privilege to acknowledge the encouragement given to me by Prof. William Seifriz (Philadelphia) during my stay abroad and later.

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COMPLEX CELLULAR STRUCTURE IN BACTERIA

K. A. BISSET and C. M. F. HALE

Department of Bacteriology, University of Birmingham, England

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Some of the errors in interpretation of cytological appearances in bacteria that have arisen from failure to recognize the complex, septate structure which is found in many bacteria were explained in a recent paper in this journal (2). This failure has to some extent been due to the relative difficulty of demonstrating the cell wall and the cross-walls or septa. In preparations stained by the routine bacteriological methods the external wall is unstained and invisible, and the internal cross-walls, which subdivide the bacillus into small cells, are usually masked by the strongly-staining, basophilic cytoplasm.

Similarly, in electron micrographs the cell wall may be recognizable as such, but its internal projections are seen only when it is disrupted and empty, and are almost indistinguishable in intact organisms.

Much valuable work has been done by means of the tannic acid-crystal violet technique, which was first popularized by Robinow (7). The tannic acid has the double effect of mordanting the cell wall to render it more readily stainable, and of destroying the cytoplasmic proteins which otherwise obscure the cross-walls. Unfortunately this method has the defect that the degree of mordanting and staining required in different cases varies very considerably, so that whereas the septa of most species of *Bacillus* can readily be demonstrated after 10 minutes in 10 per cent tannic acid at 20° C., those of *Mycobacterium* may require several hours at 60° C. to render them stainable, even after preliminary defatting treatments. These differences, in conjunction with the false staining of the cell wall which may appear in inadequately mordanted bacteria, because of the accretion of tannic acid on their surfaces, can prove very misleading to inexperienced observers.

In this laboratory it has been found that a very clear demonstration of the cell wall and cross-walls can be obtained by mordanting wet, unfixed smears in phosphomolybdic acid (4) and this paper is intended to show some of the very striking effects which can be achieved by the use of this technique, and to underline the importance of the cell envelopes in bacterial cytology.

METHODS

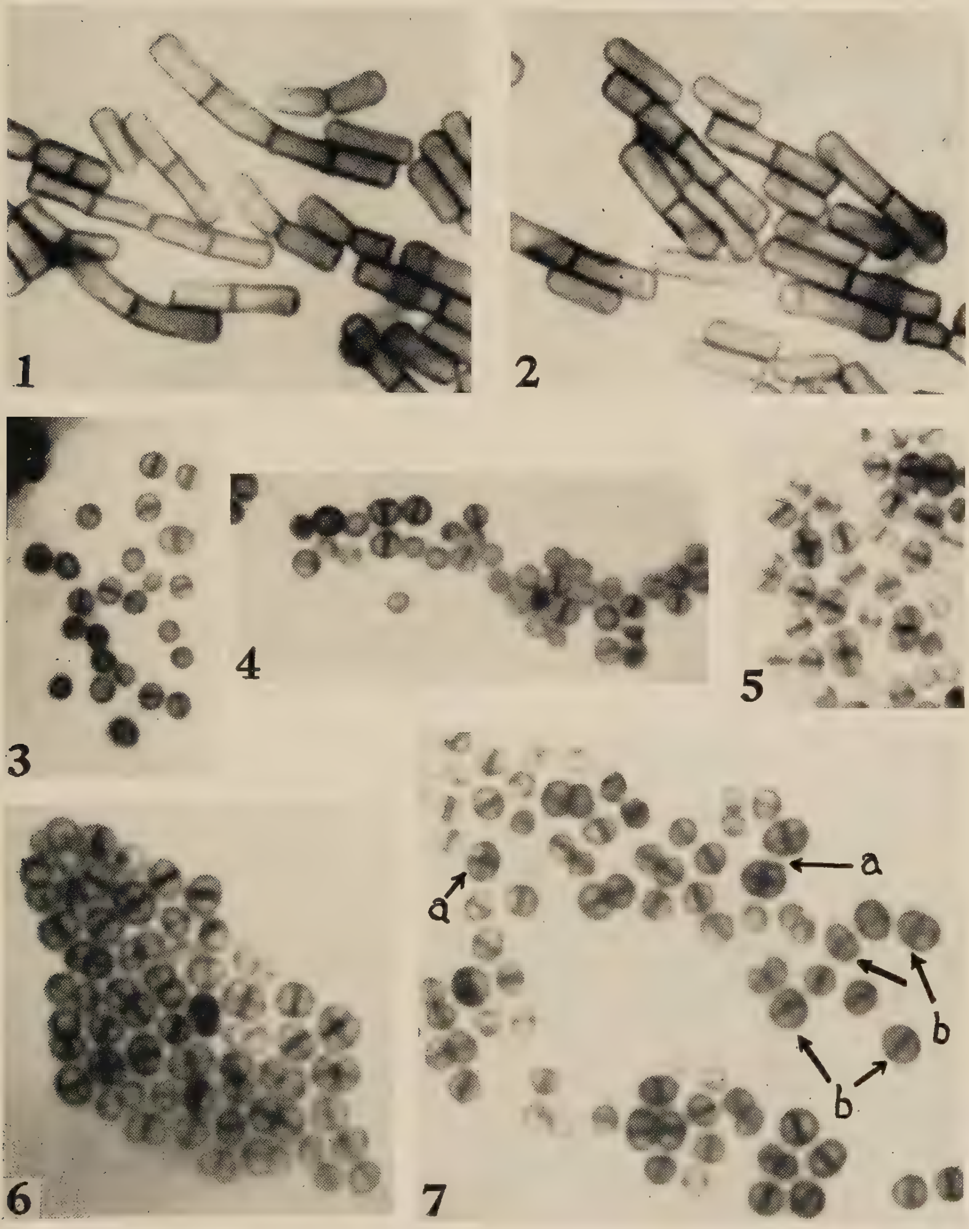
Smears, usually of cultures less than 24 hours old, were made on cover slips, rather thickly, to allow for the loss of a proportion of the material by washing, and were immersed in 1 per cent phosphomolybdic acid at room temperature (20° C. approximately) for three to five minutes, and stained with 1 per cent methyl green (Hopkins & Williams, 684) for a similar period. The cell walls stained dark green or purple, and the cytoplasm was unstained. The effect of mordanting in phosphomolybdic acid appears to be exactly comparable to that produced by tannic acid, but this method differs markedly from the tannic-acid-violet technique in that these times are not at all critical, and reasonably clear results can be obtained with a very wide variety of bacteria by the use of this standard treatment. In many cases, clearer preparations can be made by a more careful adjustment of times and temperatures, but, unlike other cell wall stains, this technique is capable of giving a sound indication of bacterial structure without any preliminary experimentation to determine the idiosyncracies of the material. A variety of other stains including crystal violet, fuchsin, brilliant green, light green and methyl blue were used experimentally but none of these produced such clear results as methyl green. Phosphotungstic acid was found to be much less effective than phosphomolybdic.

OBSERVATIONS

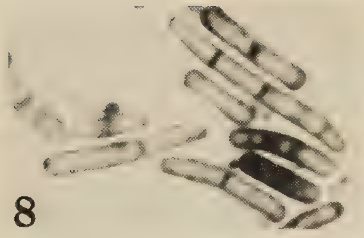
The most characteristic appearance of the cell walls in multicellular, Gram-positive bacteria is shown in Figs. 1 and 2, of *Bacillus mycoides*; each bacillus is divided into two or four units by complete cross-walls. It should be borne in mind that these units are further subdivided by cytoplasmic septa which precede and secrete the cross-walls, but which do not stain by this method, as initially they have no cell wall component (1, 7).

In micrococci a similar condition is seen, modified by the radially symmetrical habit of these organisms, so that each cross-wall is formed at right-angles to its predecessor. In Figs. 3 and 4 a strain of *Staphylococcus albus* is shown; most cocci are divided into two units by a single cross-wall, whereas in Figs. 5, 6 and 7, of a yellow-pigmented *citreus* type, the multicellularity is very marked, and a significant proportion of cocci (*a* in figure 7) have four obvious cells and may be presumed to be further subdivided both by complete cross-walls in the plane of the photomicrograph and by unstained cytoplasmic septa, representing incipient cell walls. In some organisms (*b* in Fig. 7) the second cross-wall, at right-angles to the first, can be seen at an early stage of formation.

All preparations are mordanted with phosphomolybdic acid and stained with methyl green. It is important to consider that only mature cross-walls are shown by this technique, and that nearly all these bacteria must be presumed to be even further subdivided by cytoplasmic septa (*c.f.* Bisset (1, 2), Robinow (7)). All figures are at × 2500 except 5, 6, 7, at × 3300 and 13, 17, 18 at ×1700.



Figs. 1, 2. *Bacillus mycoides*, showing typical, symmetrical formation of cross-walls.
Figs. 3, 4. *Staphylococcus albus;* most cocci are divided by a single cross-wall.
Figs. 5, 6, 7. *Staphyloccocus citreus;* some cocci are divided by a single cross-wall and some by two at right-angles (*a* in Fig. 7). In many the second cross-wall can be seen in process of formation (*b* in Fig. 7).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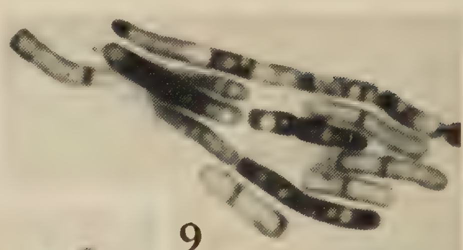
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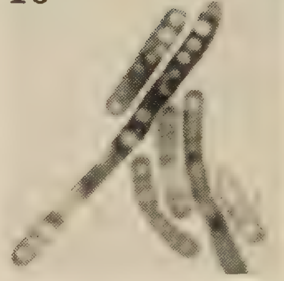
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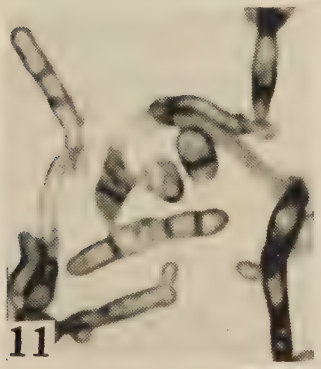
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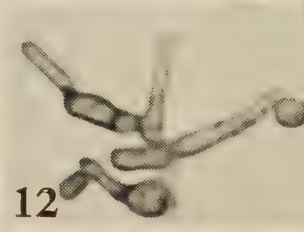
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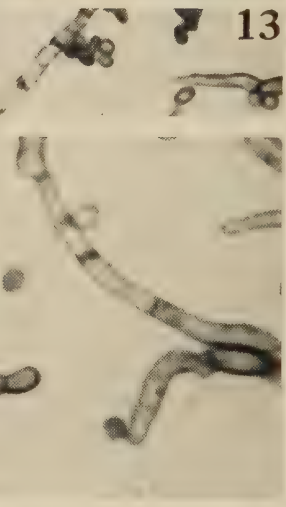
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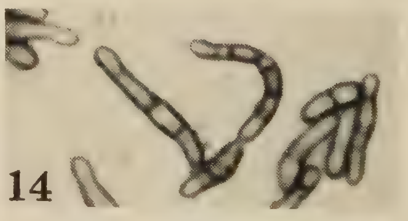
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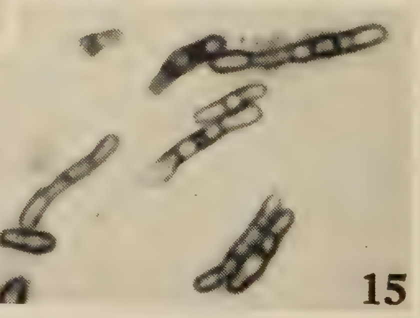
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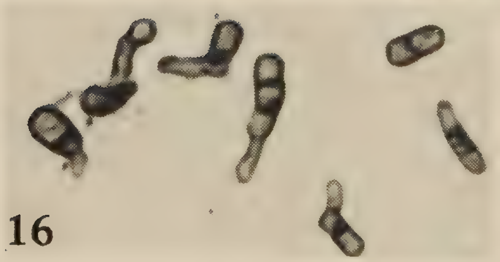
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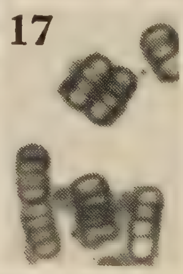
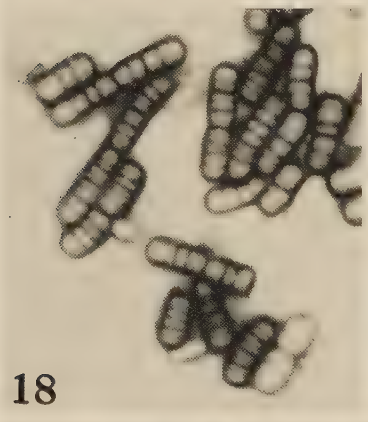

17


18

Some degree of multicellularity in cocci has already been recorded (1), but its full extent is made evident for the first time by this technique.

Different degrees of multicellularity can be seen in *Bacillus* species also. In Fig. 8, *B. megatherium*, which is a somewhat pleomorphic organism, shows the characteristic morphology of the genus. In Figs. 9 and 10, however, are seen bacilli of this species with numerous small cells, divided by relatively thick cross-walls.

The septate structure of the *Actinomycetales* is of a rather less symmetrical type than in the preceding genera of *Eubacteriales*. The morphology of *Nocardia rhodnii* is shown in Figs. 11, 12 and 13; some bud-like branches are seen, and the main filaments are subdivided rather irregularly by cross-walls. The spores, shown at a lower magnification in Fig. 13, by contrast with the true cocci are internally undivided. These observations are in accordance with the cytological description of *Nocardia* by Morris (6).

In the case of *Corynebacterium* and *Mycobacterium* especially, the demonstration of the cross-walls is greatly simplified by the use of phosphomolybdic acid; *C. diphtheriae* is shown in Figs. 14 and 15, and *M. lacticola* in Fig. 16, and the high degree of multicellularity in these bacteria (*c.f.* also (1)) is very clearly seen. The various types of granules described in both genera are seen to correspond to entire, tiny constituent cells.

An exceptional Gram-negative genus, which shows an even more marked development of transverse septa and cross-walls than do the Gram-positives is *Caryophanon*. This structure, in which the component cells are actually disc-shaped, like a pile of coins, was described by Pringsheim and Robinow (8) and is seen in Figs. 17 and 18, in *C. latum*.

DISCUSSION

By the use of a new technique for the demonstration of bacterial cell walls, involving the employment of phosphomolybdic acid as a mordant, the septate, multicellular structure of a number of representative bacteria,

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Figs. 8, 9, 10. *Bacillus megatherium;* showing typical, symmetrical formation of the cross-walls in Fig. 8, compared with extreme, irregular multicellularity in Figs. 9, 10.

Figs. 11, 12, 13. *Nocardia rhodnii;* showing irregular cross-walls and some branching. Spores are internally undivided.

Figs. 14, 15. *Corynebacterium diphtheriae;* showing irregular multicellularity with the swollen terminal cells (the "metachromatic granules" typical of the genus).

Fig. 16. *Mycobacterium lacticola;* the shape is less regular than in *M. tuberculosis*, but the numerous cross-walls are exceptionally well seen. Preparations of this genus are easier to stain after treatment with fat solvents.

Figs. 17, 18. *Caryophanon latum;* a large Gram-negative bacterium, composed of numerous disc-shaped cells, separated by cross-walls.

and especially Gram-positive genera, has been demonstrated. This confirms a number of previous observations, made by means of the tannic acid-crystal violet method, and indicates an even greater degree of complexity in some cases than had previously been understood. For example, species of coccus are shown characteristically to have no fewer than four cells per coccus, and potentially eight or more. Among the sporing bacilli *B. megatherium* may have a large, almost indefinite number of cells in each bacillus; a degree of multicellularity which is exceeded only by *Caryophanon latum*, a rather aberrant Gram-negative species.

It is highly significant that these are exactly the genera and species which the protagonists of "mitochondria" and "mitotic spindles" in bacteria persist in treating as single cells. A detailed analysis of some of these claims is given elsewhere (3).

Fortunately, this method of staining the cell walls and cross-walls of bacteria is so simple and easy to perform, and gives results of such unmistakable clarity, that its use should promote a much wider understanding of the complex cellular structure of the Gram-positive genera, and resolve many of the present discrepancies of interpretation.

At the same time, the staining of cell walls by means of a dye, methyl green, supposedly highly specific for DNA (5), emphasises the danger of interpreting cellular inclusions in bacteria by too uncritical a reliance upon such dubious specificities. This has been and remains a surprisingly common error in cytological work.

SUMMARY

A technique is described for the demonstration of bacterial cell walls, by the use of phosphomolybdic acid and methyl green. Its advantages are explained. By use of this method the complexity of the cellular structure of most Gram-positive and some Gram-negative bacteria is clearly seen, and proves to be even greater than had previously been suspected; as for example in large micrococci which may have four or more cells per coccus.

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NUCLEAR DNA CONSTANCY: A CRITICAL EVALUATION OF SOME EXCEPTIONS REPORTED BY LISON AND PASTEELS

M. ALFERT and H. SWIFT

Department of Zoology, University of California, Berkeley, Calif., and Whitman Laboratory, University of Chicago, Chicago, Ill., U.S.A.

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In recent years an increasing number of investigators have studied the amounts of desoxyribose nucleic acid (DNA) in single nuclei by microabsorption measurements of Feulgen stained tissue sections. The results have indicated that the amount of DNA per chromosome *set* in plant and animal cells is approximately constant, that DNA is doubled in amount prior to mitosis and halved in germ cell formation (1, 7, 15, 17, 18). The constancy hypothesis has sometimes been misrepresented to imply that all somatic nuclei of an organism have the same DNA content; obviously this would not be the case for the heteroploid nuclei present in many animal and plant tissues. The photometric data on DNA constancy are in agreement with biochemical studies of several workers (3, 7, 15, 22). The exactness with which DNA constancy is maintained in each nucleus is not known; means in polyploid liver nuclei match the expected 1 : 2 : 4 ratio within 2–10 per cent with standard deviations of about 10–20 per cent. Some variability can be expected from the irregularities in chromosome number known to occur in many cells (2, 6, 21) and some is also undoubtedly due to measuring technique.

Sporadically some workers have found apparent exceptions to the constancy hypothesis (4, 5, 16). This is particularly true of data obtained by Lison and Pasteels (see below) and in an attempt to reconcile these differences we have restudied a number of the tissues measured by them. As will be seen, our data and interpretations do not agree with theirs but support the concept that DNA is constant per chromosome set.

I. In measurements of rat tissues Pasteels and Lison (12) found that amounts of Feulgen dye per nucleus were approximately the same in adrenal, kidney, stomach, and lymphocyte nuclei, all averaging about half the value found for primary spermatocytes of the testis. Their measurements on liver, pancreas, and adipose tissue averaged from 15 to 30 per cent lower than those from the other tissues and they suggest that diminution of chromatin material has occurred here. As has been pointed out elsewhere (17, 19), with many fixatives the intensity of the Feulgen reaction decreases from the edge to the center of the tissue section. This is particularly marked where large blocks of tissue are used, and is less evident in loose, easily penetrated tissues such as testis. We feel that the "diminution" described by Pasteels and Lison is due to this variable rather than to any true variation in the amount of DNA. In Table I some of our values on formalin fixed tissues are compared with those of Pasteels and Lison. When the same tissues were homogenized in 30 per cent sucrose before fixing, the difference between liver and testis is no longer significant. The higher relative values obtained from homogenized nuclei are due to an increase in Feulgen intensity caused by the treatment rather than to any change in distribution of the absorbing material (11). The means of the polyploid liver nuclei given by Pasteels and Lison show a discrepancy of about 23 per cent from the expected 1:2:4 ratios as compared with a 10 per cent variation found by Ris and Mirsky (15) and Leuchtenberger *et al.* (7) and a 2 to 4 per cent variation found by Swift (17), Alfert (unpublished) and Patau and Bloch (unpublished).

Table I

Amounts of DNA in rat nuclei isolated in 30 per cent sucrose before fixation, as compared with nuclei from sections of large tissue blocks. Means and standard errors are given; numbers of measurements are in parentheses.

	Tissue sections		Sucrose isolated
	Pasteels and Lison (12)	Alfert and Swift	Alfert and Swift
Liver (4N nuclei)	347±4 (97)	[1]347±3 (15)	486±8 (15)
Testis (primary spermatocytes)	479±3 (81)	443±4 (15)	502±8 (15)

II. Amounts of DNA in mitotic stages from rat crypts of Lieberkühn have been estimated by Pasteels and Lison (13). They conclude, on the basis of these data, that DNA is reduced to half the normal amount at anaphase and is restored to normal in the following telophase or very early interphase. We have measured the same tissue, and our values are given beside those of Pasteels and Lison in Table II. With formalin fixation as used in both cases many metaphase and anaphase figures are sufficiently clumped in a homogeneous mass so that photometric measurements may be made. Our values would indicate that DNA synthesis occurs in this tissue in interphase some time before prophase begins, and not in telophase or early interphase. This pattern appears to be characteristic of dividing cells in many other, but not in all, tissues (19). In the rat crypts of Lieberkühn anaphase and telophase nuclei are small and stain very strongly. Error due to stray light would be large in most measuring instruments under these conditions (11). This error if present would give the relationship between nuclear size and extinction found by Pasteels and Lison and interpreted by them as true DNA variation, associated with synthesis. Our measurements have been made at a wavelength off the Feulgen absorption peak, to minimize the stray light error. We find two distinct classes of interphase nuclei in the intestinal epithelium, in contrast to the continuous variation found by Pasteels and Lison.

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[1] This value was set equal to the corresponding one of Pasteels and Lison; others are proportional to it.

TABLE II

Amounts of DNA in nuclei of rat crypts of Lieberkühn. Means and standard errors are given; numbers of measurements are in parentheses.

Stage	Pasteels and Lison (13)		Alfert and Swift		
Interphase	Continuous variation from 740–2620	(38)	Class I	16.2±0.34	(35)
			Class II	30.2, 32.3	(2)
Prophase	1470, 1810, 1850, 1950	(4)		23.5, 32.0, 32.0, 34.7, 37.5	(5)
Metaphase	1377±28	(21)		32.3±0.91	(29)
Anaphase	—			16.5±0.46	(24)
Telophase	Continuous increase proportional to nuclear surface, 650–1300	(23)	Early	17.0±0.42	(21)
			Mid	17.5±0.40	(26)
			Late to early interphase	16.6±0.52	(19)

III. Pasteels and Lison (13) have claimed that nuclei of dividing tissues have a higher DNA content than those in nondividing tissues since according to their calculations chick fibroblasts in tissue culture gave the results shown in Table III. Pasteels and Lison calculate amounts of dye by multiplying the extinction of a small central core of each nucleus by the total projected area of the nucleus. This product is actually proportional not to the dye content of a spherical nucleus, but to the content of its circumscribed cylinder. When *spherical* nuclei are compared to each other such a computation may be used, but each value will be too high by a constant factor of 50 per cent corresponding to the difference between the volume of a sphere and that of its circumscribed cylinder. In the present case the fibroblast nuclei in the migration zone are greatly flattened and have a roughly discoidal shape while the nuclei in the dividing zone are spherical. The calculation used will therefore give proportional values of dye content for cylindrical nuclei in the migration zone but values approximately 50 per cent too high in the dividing zone. If the latter are multiplied by $^2/_3$, the differences in shape are corrected and the values of migration zone nuclei and Class I nuclei agree well, as shown in the last column of Table III. The identical data cited in Tables II and III were published again by Lison and Pasteels (8). In this second paper, however, a different method of calculation is described, using measurements of large instead of small central nuclear cores. Since these two methods used on the same material would not produce the same result, it is difficult to see how identical data could have been obtained.

TABLE III

Amounts of DNA in chick fibroblasts in tissue culture.

	Pasteels and Lison (13)	Corrected values
Migration zone	73.6 ± 2.0	73.6 ± 2.0
Multiplication zone Class I	108.5 ± 1.8	71.6 ± 1.1
Class II	195.8 ± 4.5	129.2 ± 3.0

IV. Pasteels and Lison (14) have published data on maturation and cleavage in the annelid *Sabellaria*. Their values are reproduced in the first column of Table IV. We have measured similar stages in presumably the same species (S. *alveolata*). Our values are given in the second column of Table IV and beside them the values expected on the basis of the constancy hypothesis, C being a constant. The low sperm values found by Pasteels and Lison are possibly due to the fact that many *Sabellaria* sperm undergo partial disintegration during Feulgen hydrolysis. This phenomenon might be readily overlooked in the few sperm adhering to the eggs; it is at once apparent in sections of a mature testis. Our data show the same DNA picture found in grashopper maturation and cleavage (20). Data by Lison and Pasteels (9) on sea urchin cleavage do not show DNA constancy but recent data of McMaster (10) on another sea urchin species are in complete agreement with the constancy hypothesis.

At present the constant relationship between DNA and the chromosomes seems well established in a wide variety of tissues. Valid exceptions to this pattern would thus appear to be of considerable interest. We do not feel, however, that the extensive data of Lison and Pasteels invalidate the constancy hypothesis. The discrepancies between their results and ours are rather due, in our opinion, to errors of technique and interpretation.

TABLE IV

Amounts of DNA during maturation and cleavage of *Sabellaria aveolata*. Means and standard errors are given; numbers of measurements in parentheses.

Stage	Pasteels and Lison (14)	Alfert and Swift	Expected values
1st polar body	127 ± 3 (17)	[1]127 ± 3 (20)	2 c
Free sperm	29 ± 1 (26)	61 ± 1 (14)	1 c
Sperm, after penetration into the egg at			
a) 1st meiotic metaphase	44 ± 1 (27)	62 ± 3 (18)	1 c
b) 1st meiotic telophase	57 ± 1 (26)		
Pronuclei	177 ± 1 (18)	133 ± 7 (31)	1→2 c
Cleavage nuclei in			
a) Prophase	365 ± 4 (6)	[2]263 ± 10 (13)	4 c
b) Interphase	355 ± 5 (6)	210 ± 9 (13)	2→4 c
c) Telophase	—	124 ± 3 (14)	2 c

The *Sabellaria* material was collected and fixed for us by Dr. Howard Bern at Concarneau, France, and we are greatly indebted to Professor Fauré-Fremiet for his kind assistance in identification and preparation of the material. We also wish to thank Dr. Ellen Rasch and Mrs. Ruth Kleinfeld for help with some of the measurements for Table II. This work was supported in part by the Wallace C. and Clara A. Abbott Memorial Fund, the United States Public Health Service, and research funds of the University of California; a travel allowance from the Rockefeller Foundation made possible a visit to the laboratory of Drs. Pasteels and Lison by the senior author.

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[1] This value was set equal to the corresponding one of Pasteels and Lison; all others are proportional to it.

[2] Largest interphase and early prophase nuclei of 4-cell stages.

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CONTRIBUTION A L'ÉTUDE DES CELLULES BINUCLÉÉES DANS DES CULTURES DE FIBROBLASTES[1]

O. BUCHER et R. GATTIKER

Institut d'Histologie et d'Embryologie de l'Université de Lausanne, Suisse

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Les cellules binucléées — *in vivo* et *in vitro* — nous intéressent en ce qui concerne maints problèmes cytophysiologiques, comme par exemple la règle de Jacobj (21) sur la croissance rythmique par dédoublement de nombreuses cellules, l'amitose etc. Ainsi nous avons déjà mentionné un premier problème: comment se comportent les fréquences des grandeurs nucléaires des cellules binucléées (somme des deux noyaux) par rapport à celles des cellules à un seul noyau? La masse nucléaire (et avec elle, selon la relation nucléoplasmatique, le corps cellulaire) a-t-elle augmenté de volume ou non, et, si tel est le cas, cet accroissement se fait-il, comme l'admettent certains auteurs, par un facteur défini, par exemple 2 ou $\sqrt{2}$?

La majorité des chercheurs s'accorde de nos jours à admettre que la formation de cellules binucléées est due, d'une manière générale à l'amitose (pour la littérature, cf. Münzer (29, 30), Jacobj (21, 22), Levi (23), Pfuhl (32) et d'autres). D'autre part, on a pu démontrer que des cellules binucléées furent obtenues dans des conditions appropriées d'expérimentation, comme par exemple dans les cultures de tissus *in vitro*, à la suite de mitoses abortives, c'est-à-dire de divisions nucléaires sans clivage cellulaire consécutif (3).

Si le fait de posséder deux noyaux est la conséquence d'une mitose inachevée, il faut supposer que chacun d'entre eux renferme le même lot chromosomique, et possède donc le même volume. Cependant, si la division directe en est la cause, nous ne pouvons accepter cette propriété de prime abord. Quant à la connaissance du comportement des noyaux produits par une division amitotique, nous n'avons trouvé, en ce qui concerne leur taille, aucune autre indication précise dans la littérature. A notre avis, il est possible que les deux noyaux — s'ils n'ont pas la même grandeur — soient tout de même dans un rapport défini, c'est-à-dire que le volume du plus petit noyau divisé par celui du plus grand donne un quotient plus ou moins constant.

La plupart des recherches consacrées au problème esquissé furent effectuées sur des coupes, et tout particulièrement des préparations de foie, où il

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[1] Avec l'appui de l'Académie Suisse des Sciences Médicales.

faut compter avec certaines fautes de méthode (voir Pfuhl, (31)), puisque, dans les préparations histologiques, deux noyaux ne peuvent se rencontrer sous leur plus grand diamètre que dans un cas qui serait idéal. Il s'impose donc de procéder à de telles recherches sur des préparations totales de tissus cultivés *in vitro* (cultures sur lamelles). Si de tels travaux n'ont guère été effectués, c'est parce qu'il fallait d'abord mettre au point une méthode appropriée aux recherches caryométriques quantitatives (10–13).

Ainsi il nous semble intéressant de reprendre quelques problèmes concernant les cellules binucléées et d'utiliser, en guise de matériel d'expérimentation, des cultures de fibroblastes de différents animaux et de l'homme. En passant nous aborderons aussi la question de la fréquence des cellules bi- et plurinucléées dans des cultures non traitées et nous envisagerons la possibilité d'influencer cette fréquence par quelques applications expérimentales (cf. (5, 7, 8, 9, 17)).

MATÉRIEL ET MÉTHODE

Notre matériel d'expérimentation se compose de cultures de fibroblastes (et d'ostéoblastes) sur lamelles provenant de divers animaux — poulet, souris, lapin, cobaye — et de l'homme (*cf.* tableau I). Pour la présente communication, plus de 36000 cellules furent mesurées au planimètre; parmi celles-ci, nous trouvons, dans les cultures non influencées, 407 cellules binucléées ainsi qu'une cellule à trois et

Tableau I

Vue d'ensemble sur les noyaux évalués pour nos recherches.

Espèce animale	Noyaux des cultures non influencées	Noyaux des cultures influencées
Poulet:		
a) Fibroblastes:		
1) Explantats cutanés.......	1650	
2) Explantats cardiaques	3700	
b) Ostéoblastes	1000	
Fibroblastes de souris	3700	
Fibroblastes de lapin:		
a) $2^{\text{ème}}$ jour	6200	13450
b) $4^{\text{ème}}$ jour	4500	
Fibroblastes de cobaye	1000	
Fibroblastes humains	1000	
Total	22750	13450

une à quatre noyaux; dans le matériel influencé expérimentalement 182 cellules à deux et 8 à trois noyaux. Nous disposons donc de 589 cellules binucléées dont les noyaux furent évalués quantitativement.

Comme nous l'avons montré dans nos travaux précédents, la grandeur nucléaire dans l'auréole de croissance des cultures de fibroblastes n'est pas uniforme : dans la zone périphérique (zone marginale) les noyaux ont en moyenne un volume double de ceux de la zone interne contiguë à la pièce souche (10); les deux zones se fondent graduellement l'une dans l'autre. Nous nous sommes limités au matériel nucléaire de la zone externe, puisque l'union cellulaire est ici plus lâche, et que l'on distingue plus facilement les cellules binucléées.

Cependant, nous avons étudié le matériel nucléaire de la zone interne et trouvé, d'une manière générale, un pourcentage plus faible de cellules bi- et plurinucléées. Nous ne pouvons établir dans le cadre de ce travail si ce résultat s'explique uniquement par la méthode employée, ou encore, comme nous sommes enclins à le penser, par un fait biologique.

Nous ne reviendrons pas sur la méthode employée pour nos recherches caryométriques puisque nous l'avons décrite et justifiée dans nos publications précédentes (4, 6, 13); pour le dépouillement statistique des chiffres obtenus empiriquement cf. surtout Bucher et Gattiker (12). La difficulté principale dans la mesure nucléaire *in vitro* — et dans le tissu conjonctif en général, *in vivo* aussi — réside dans le fait que le noyau cellulaire ne présente pas une forme géométrique définie (telles la sphère ou l'ellipsoïde de révolution) comme la grande majorité des noyaux mesurés dans des coupes histologiques, mais se trouve être fortement aplati. Le troisième diamètre nucléaire perpendiculaire à la lamelle ne se laisse pas déterminer dans les préparations totales; par conséquent, nous ne pouvons pas non plus évaluer le volume absolu. Comme celui-ci nous intéresse beaucoup moins que les rapports entre les grandeurs nucléaires, il nous est possible d'aboutir à un résultat de manière plus simple. En effet, il s'est révélé que le troisième diamètre varie proportionellement aux deux autres (c'est-à-dire aussi à la surface de projection planimétrée), ce qui permet de l'éliminer mathématiquement. Si le *volume* nucléaire a doublé, le plan de projection que nous avons mesuré a augmenté d'un facteur de $1{,}5875 - \sqrt{2^2}$ (6, 13) cependant, si la *surface* nucléaire a doublé, la valeur planimétrique obtenue s'est agrandie de deux fois. De cette manière, nous disposons des bases mathématiques qui vont nous permettre de discuter, en nous fondant sur l'évaluation des plans de projection dessinés, le comportement relatif aussi bien du volume que de la surface nucléaires.

Nous avons attribué une importance toute particulière au dépouillement statistique des chiffres des mensurations, comme on peut également le relever en détail dans un de nos travaux précédents (12).

RÉSULTATS DE NOS RECHERCHES

Des recherches sur des coupes d'organes (foie) montrèrent que différentes espèces animales présentaient un pourcentage inégal de cellules binucléées et que, pour une espèce donnée, ce pourcentage était approximativement constant. Pour cette raison nous nous sommes d'abord demandés si on pouvait établir, dans des cultures de fibroblastes de divers animaux, un pourcentage différent de cellules bi- et plurinucléées. Nos résultats sont réunis dans le tableau II. Nous nous rendons compte que c'est seulement en présence d'un matériel nucléaire suffisamment grand que l'on ose tirer des conclusions significatives et que des variations dans les conditions de culture peuvent également influer sur le résultat.

TABLEAU II

Fréquences des cellules bi- et plurinucléées.

Espèce animale et tissu	Noyaux mesurés	Fréquences des cellules binucléées		Cellules binucléées à noyaux de même taille (% des cellules binucléées)	Cellules plurinucléées
		en chiffres absolus	en ‰		
Fibroblastes de lapin:					
2ème jour	6200	88	14,2	21,5	1 à 3 noyaux
4ème jour	4500	63	14,0	26,9	1 à 4 noyaux
Fibroblastes de lapin:					
Trypaflavine 1:600.000	1500	39	26,0	16,2	2 à 3 noyaux
Traitement préalable à la Colchicine	1800	35	19,4	25,7	4 à 3 noyaux
Fibroblastes de poulet (explantats de peau et de cœur)	5350	115	21,4	25,2	
Ostéoblastes de poulet	1000	38	38,0	34,2	
Fibroblastes de souris	3700	69	18,6	17,3	
Fibroblastes de cobaye	1000	22	22,0	27,3	
Fibroblastes humains	1000	12	12,0	16,7	

Ainsi, par exemple, nous avons trouvé dans une première série de cultures de fibroblastes de souris 38 (26,2 ‰) cellules binucléées parmi 1450 cellules évaluées par caryométrie, et dans une deuxième série de 2250 cellules, seulement 31 (13,8 ‰). Nous ne voulons donc pas trop insister sur les pour mille indiqués à propos des cultures de tissu conjonctif de cobaye et d'homme, les valeurs en question ne se basant que sur l'évaluation de mille cellules de chaque espèce. De ce que nous venons de dire il résulte que, lors d'influences expérimentales, il faut faire le plus attention possible, pour que les cultures traitées et les témoins correspondants soient soumis à des conditions strictement égales et que l'on utilise pour les essais en question des cultures de qualité approximativement identique.

Il ressort du tableau II que, dans la zone externe des cultures non influencées de fibroblastes de lapin (aussi bien du 2ème jour que du 4ème), environ

14 ‰ des cellules sont binucléées. Nous trouvons un pourcentage sensiblement plus élevé dans les cultures de fibroblastes de poulet (21,4 ‰), et particulièrement dans celles d'ostéoblastes de poulet (38,0 ‰).

Il est intéressant de noter le pourcentage augmenté des cellules à deux noyaux dans les cultures de fibroblastes de lapin qui furent exposées pendant environ 9 heures à l'influence de la trypaflavine à 1:600000, ainsi que dans les cultures préalablement traitées à la colchicine (1:10 millions ou 1:20 millions). Nous reviendrons sur l'interprétation de ces observations dans la section consacrée à la discussion des résultats.

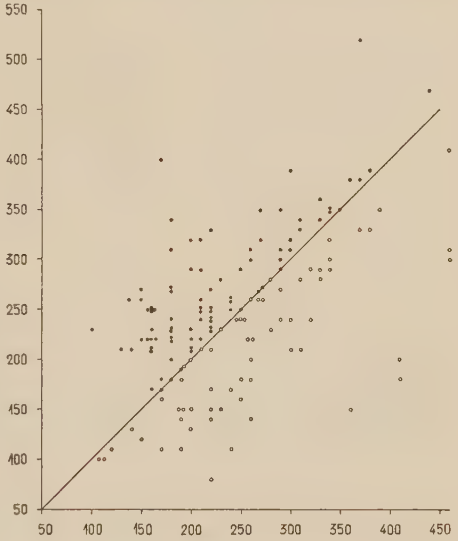

Fig. 1. Diagramme de dispersion des fibroblastes binucléés de lapin, compte tenu de la grandeur de chacun de leurs noyaux: chaque paire nucléaire est représentée par un point, dont l'abscisse désigne la valeur planimétrique de projection de l'un des noyaux, et l'ordonnée celle de l'autre. Ces points tombent sur la bissectrice, si les deux noyaux de la paire ont même taille. Les points placés au-dessus de la bissectrice correspondent aux résultats tirés des cultures du deuxième jour, les cercles en-dessous à ceux des cultures du quatrième jour.

Nous pouvons prendre position avec une plus grande certitude sur le problème suivant: les noyaux d'une même paire, dans des cellules binucléées, sont-ils égaux ou inégaux? Et, dans ce dernier cas, *existe-t-il une relation quantitative définie entre les deux noyaux?*

Pour nous faire une première idée sur cette question, nous avons porté dans le système de coordonnées (fig. 1) chaque paire nucléaire évaluée planimétriquement dans des cultures de fibroblastes de lapin de telle manière que la grandeur de l'un des noyaux soit représentée par l'abscisse, celle de l'autre par l'ordonnée. Si les noyaux ont la même taille, le point définissant la paire correspondante se situe sur la bissectrice de notre système. Nous avons porté les noyaux des cellules binucléées des cultures du deuxième jour au-dessus de la bissectrice, ceux des cultures du quatrième jour audessous. On constate que la densité des points situés des deux côtés de la bissectrice diminue de manière continue à mesure que l'on s'en éloigne. Nous obtenons un résultat identique, dans son principe, par la représentation des cellules binucléées des cultures de tissu conjonctif d'autres animaux et de l'homme, ce qui nous permet de renoncer à la reproduction des figures s'y rapportant.

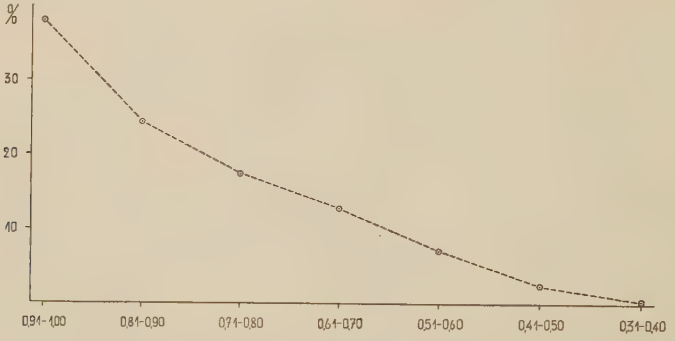

Fig. 2. Distribution de fréquences des quotients de grandeurs nucléaires de 369 fibroblastes binucléés. Sur l'abscisse sont portés les quotients, sur l'ordonnée les fréquences correspondantes (en %).

On rencontre une grandeur plus ou moins identique des deux noyaux, comme nous le montre le tableau II, dans le 16–34 % des cellules binucléées, autrement dit dans le 24,8 % en moyenne des 407 couples nucléaires étudiés dans des cultures de fibroblastes non influencées expérimentalement. Les variations indiquées ci-dessus sont conditionnées fort probablement moins par la provenance diverse du tissu que, en tout cas partiellement, par le nombre restreint des noyaux mesurés.

Sont considérés comme égaux, les noyaux d'une paire dont les plans de projection ne diffèrent pas de plus de 10 mm². Cette marge, que nous avons dû adopter, correspond à l'erreur de notre méthode, pour laquelle Bucher et Horisberger (13) ont calculé un écart-type de s = 6,4 mm². Pour de plus amples détails, voir Gattiker (17).

Dans le but d'obtenir un critère quantitatif pour indiquer la différence de grandeur des deux noyaux des cellules binucléées, nous avons calculé le *quotient petit noyau sur grand noyau.* Comme l'ensemble du matériel des fibroblastes *in vitro* montrait à ce point de vue un même comportement, nous avons réuni pour le dépouillement statistique les résultats des 369 couples nucléaires et réparti les quotients dans des classes d'une largeur de 0,1. La courbe qui en résulte est représentée dans la fig. 2. Les quotients

TABLEAU III

Quotient moyen (petit noyau sur grand noyau) des grandeurs nucléaires des cellules binucléées. Les chiffres entre paranthèses de l'entête du tableau désignent le nombre des cellules à deux noyaux que nous avons évalués à cette intention.

Classes de grandeur nucléaire	Poulet			Fibroblastes de souris	Fibroblastes de lapin		Fibroblastes de cobaye	Fibroblastes humains	Quotient moyen
	Fibroblastes		Ostéoblastes						
	explantat de peau (85)	explantat de cœur (30)	(38)	(69)	2ème jour (88)	4ème jour (63)	(22)	(12)	(407)
195– 215	0,79	0,70	1,00	0,75		0,94			0,87
220– 240	0,83	0,68	0,82	0,92		0,92			0,78
245– 270	0,80	0,87	0,80	0,86		0,81			0,81
275– 305	0,83	0,81	0,80	0,90		0,64			0,79
310– 340	0,81	0,88	0,87	0,88	0,76	0,63	0,93		0,82
345– 385	0,86	0,80	0,82	0,80	0,82	0,76	0,90	0,75	0,82
390– 430	0,84	0,88	0,69	0,83	0,79	0,78	0,82	0,75	0,81
435– 485	0,70	0,78	0,86	0,82	0,82	0,84	0,88	1,00	0,83
490– 545	0,86		0,96	0,80	0,83	0,83	0,85	0,78	0,80
550– 610			0,94	0,81	0,86	0,83	0,85	0,82	0,82
615– 685			0,98	0,81	0,92	0,84	0,94	0,79	0,88
690– 770				0,82	0,90	0,84	0,82	0,91	0,83
775– 865				0,84	0,92	0,76	0,93		0,86
870– 970				0,94	0,83	0,84	0,94		0,85
975–1090				0,82		0,88			0,87
1095–1225				0,82					0,82
	0,82	0,82	0,84	0,82	0,83	0,80	0,88	0,82	0,82

sont portés en abscisse, les fréquences correspondantes en ordonnée. La plus grande fréquence se trouve dans la classe des quotients allant de 0,91 à 1,00; à mesure que les quotients décroissent les fréquences diminuent graduellement. D'où la conclusion que les deux noyaux d'une cellule binucléée montrent une certaine tendance à adopter la même grandeur.

Il est logique de se poser maintenant la question suivante: existe-t-il une *relation entre les quotients et la masse des deux noyaux* d'une cellule binucléée? C'est pourquoi, comme dans nos travaux précédents (10, 12), nous avons réparti les grandeurs nucléaires (somme des deux noyaux d'une paire), avec leur quotient, dans un système de classes à échelle logarithmique, et calculé le quotient moyen pour chaque classe de grandeur nucléaire. Le résultat (comparer tableau III) est frappant: tous les tissus examinés ainsi que toutes les classes de grandeurs révèlent — compte tenu des variations que l'on est en droit d'attendre au cours de recherches biologiques sur un matériel relativement petit — un quotient à peu près constant. Aussi bien les quotients moyens, indiqués dans la rangée inférieure du tableau et se rapportant à divers tissus et à différents animaux, que le quotient moyen par classe de grandeurs nucléaires, mentionné dans la dernière colonne de droite, ne diffèrent que très peu. Au point d'intersection des deux colonnes mentionnées dans le tableau III se trouve le quotient moyen de 0,82 calculé à partir des 407 valeurs obtenues.

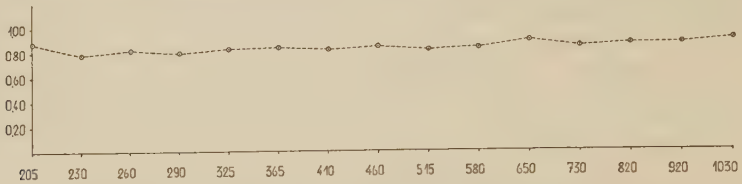

Fig. 3. Représentation des quotients des grandeurs nucléaires petit noyau sur grand noyau (portés en ordonnées) de fibroblastes binucléés en fonction des tailles nucléaires (portées en abscisses). On reconnaît la constance de ces quotients au parallélisme entre la ligne de succession des points et l'abscisse.

Dans la fig. 3 nous avons porté en abscisse les grandeurs des couples nucléaires (plans de projection évalués planimétriquement), en ordonnée les quotients moyens correspondants calculés de la manière mentionnée ci-dessus. En réunissant les points dessinés on se trouve en présence d'une ligne approximativement droite et parallèle à l'abscisse; *nous avons donc démontré que le quotient des volumes nucléaires des cellules binucléées est constant et indépendant de la grandeur absolue du couple nucléaire.*

Comme, ainsi que nous venons de le constater, le quotient des grandeurs nucléaires est pratiquement invariable, il est évident que la différence entre deux noyaux d'une paire doit augmenter proportionnellement à la masse nucléaire totale de la cellule; cela ressort également des chiffres empiriques de notre matériel d'essai.

Dans les cultures intoxiquées à la colchicine ou à la trypaflavine le quotient varie dans le même ordre de grandeur (il montre néanmoins des oscillations un peu plus grandes après un traitement préalable à la colchicine).

Comment se présente alors la masse nucléaire des cellules binucléées par rapport à celle des cellules à un noyau? A cet égard nos résultats ne sont pas concordants. Dans la fig. 4 a nous avons opposé la courbe des fréquences des grandeurs nucléaires, établie à partir des 230 noyaux des cellules binucléées pris séparément, et celle tirée des 115 couples nucléaires des cultures de fibroblastes de poulet, aux deux courbes de variation des cellules uninucléées des zones interne et externe (dont les maxima correspondent respectivement à K_1 et à K_2). On reconnaît que le milieu de la distribution de fréquences des grandeurs de chaque noyau d'une cellule binucléée tombe sur K_1 et que celui des grandeurs des deux noyaux pris ensemble correspond à K_2. La moyenne des volumes nucléaires des cellules binucléées concorde en principe avec celle des volumes des cellules à un noyau de la zone externe, tandis que la moyenne des noyaux d'une paire, chaque noyau pris séparément, se comporte comme celle des tailles nucléaires des cellules à un noyau de la zone interne. Par conséquent, les cellules binucléées de nos fibroblastes de poulet cultivés *in vitro*, ne possèdent pas un matériel nucléaire plus abondant que les autres cellules de la même zone. Nous obtenons un résultat analogue dans les cultures d'ostéoblastes de poulet.

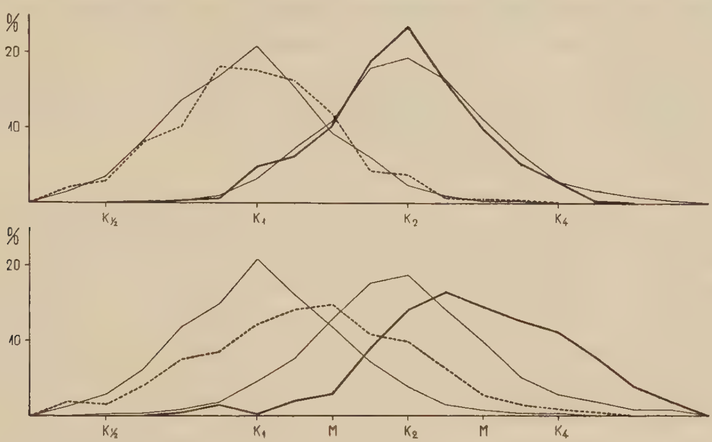

Fig. 4. a) Courbes de fréquences des grandeurs nucléaires de fibroblastes de poulet. Les courbes en trait fin représentent le comportement des noyaux des cellules uninucléées des zones interne (à gauche) et externe (à droite) de l'auréole de croissance, chaque courbe se basant sur l'évaluation planimétrique de 2250 noyaux. En pointillé: courbe obtenue à partir de 230 noyaux (pris séparément) de cellules binucléées; en trait gras: courbe calculée sur la base de 115 paires nucléaires (sommes des deux volumes) de ces mêmes cellules binucléées de la zone externe. b) Courbes de fréquences des grandeurs nucléaires de fibroblastes de lapin. En trait fin, courbes représentant le comportement d'environ 8000 noyaux de cellules uninucléées des zones interne et externe (respectivement à gauche et à droite); en pointillé: courbe fondée sur l'évaluation de 304 noyaux (pris isolément) de cellules binucléées; en trait gras: courbe construite sur la base de 152 couples nucléaires (somme des deux volumes) des mêmes cellules binucléées de la zone externe.

Cependant, dans nos cultures, les fibroblastes binucléés de lapin se comportent tout différemment: les cultures du deuxième et du quatrième jour, menant en principe aux mêmes résultats, nous avons réuni ceux-ci pour obtenir la fig. 4 b, ce qui met à notre disposition un plus grand nombre de chiffres. On reconnaît que le milieu de l'intégrale des courbes de fréquences des grandeurs nucléaires, concernant aussi bien les noyaux des cellules binucléées pris séparément que les sommes des deux partenaires nucléaires, ne concordent pas respectivement avec K_1 ou K_2 mais approximativement avec la classe moyenne M supérieure. Dans ce cas, les cellules binucléées possèdent par conséquent un caryoplasme qui, comparé à celui des cellules uninucléées de la zone correspondante (zone extérieure), a augmenté du facteur $\sqrt{2}$. Nos cultures de fibroblastes de cobaye et d'homme présentent à ce point de vue les mêmes propriétés.

A propos de la méthode utilisée, nous voudrions encore rappeler que nous ne travaillons pas avec les volumes nucléaires eux-mêmes mais avec les plans de projection. Comme nous l'avons déjà mentionné dans la division «matériel et méthode», nous savons qu'ils sont dans le rapport de 1,5875, à condition que les volumes correspondants soient dans celui de 1 : 2 (pour les détails voir O. Bucher et B. Horisberger, (13)). Nous pouvons discuter la somme des volumes d'un couple nucléaire à condition seulement de diviser les sommes des valeurs des surfaces planimétrées par 2 et de les multiplier par notre facteur 1,5875. Il est inadmissible de les additionner simplement.

Nous avons examiné nos cellules binucléées non seulement quantitativement, mais encore qualitativement, c'est-à-dire sous leur aspect morphologique. A cette occasion, les deux noyaux d'une paire montrèrent presque toujours la même forme; en ce qui concerne leur colorabilité, nous ne pûmes constater aucune différence notable. Par conséquent, les deux noyaux paraissent équivalents et ni l'un ni l'autre ne montre dans nos cultures une tendance à la dégénérescence. La substance nucléolaire, elle aussi, se répartit en général sur les deux noyaux d'une manière plus ou moins égale et se comporte d'une façon très semblable, dans bien des cas, quant à la forme et la disposition.

DISCUSSION DES RÉSULTATS

Dans les cultures de fibroblastes de poulet examinées, on a pu constater environ 21 ‰ de cellules binucléées (voir aussi tableau II), donc passablement davantage que Macklin (25), Zweibaum et Szejnman (36) qui trouvèrent dans le même tissu respectivement 9 et 7 ‰. Il se pourrait que, dans le détail, des différences de conditions de cultures puissent jouer un certain rôle. Levi (23) aussi et d'autres, mentionnèrent que dans des circonstances moins favorables pour les cultures, le nombre des divisions directes, et par conséquent celui des cellules binucléées augmentait. Nous trouvâmes un pourcentage de telles cellules dans les cultures de fibroblastes de lapin plus faible (environ 14 %) que dans celles de poulet; sur ce sujet la littérature ne nous fournit aucune indication. Il serait intéressant de savoir si les différences de fréquences des cellules bi- et plurinucléées dans des cultures de tissu conjonctif de provenances diverses étaient spécifiques de l'espèce des animaux en question, comme le montra Münzer (29) pour les cellules hépatiques *in vivo*. Lors d'expériences sur des tissus cultivés *in vitro*, il serait éventuellement encore indiqué de noter si on a fait un explantat d'un fœtus ou d'un animal jeune ou âgé. Ainsi l'auteur précédemment cité put constater — de nouveau sur du foie — que les cellules binucléées sont plus rares chez le foetus qu'après la naissance, et qu'elles augmentent avec l'âge chez tous les mammifères examinés.

Si l'on se renseigne dans la littérature sur le rapport de grandeur des deux noyaux des cellules binucléées, l'opinion, qui dans la plupart des cas ne repose que sur des estimations vagues et selon laquelle les noyaux auraient le plus souvent les mêmes dimensions est nettement prédominante (cf. 29, 21, 35, 31, 14). Gauer (18) s'attacha d'un peu plus près à cette question; pour l'épithélium de transition des voies urinaires humaines, il indiqua que, dans le 33 % des cas, les deux noyaux d'une cellule binucléée se disposaient dans la même classe de grandeur de son système de répartition, dans le 47 %, dans une classe voisine (proportion de ces noyaux 5 : 6), et que le 3 % des deux noyaux des cellules binucléées étaient dans la relation de 1 à 2. Si nous appliquons cette même façon de juger à 151 fibroblastes binucléés *in vitro* de lapin, le 29,6 % des couples nucléaires tombent dans la même classe, le 22,4 % dans des classes voisines; un rapport de volume de 1 à 2 se rencontre dans 9 % des cas en chiffres ronds. Il va sans dire que pour des raisons biologiques et statistiques (cf. (12)) l'on doit adopter pour faire cette répartition un système logarithmique de classification.

Nous acquérons des connaissances plus poussées au sujet des rapports dimensionnels entre les deux noyaux d'une paire en calculant, comme nous l'avons proposé, le quotient petit noyau sur grand noyau (voir fig. 2 et 3, ainsi que tableau III). Le diagramme 2 montre que la fréquence augmente d'autant plus que les deux noyaux des cellules binucléées diffèrent moins. Ce résultat concorde en principe avec la conception prédominante dans la littérature, conception suivant laquelle, d'une manière générale, les deux grandeurs nucléaires ne diffèrent pas de façon appréciable.

Elle nous paraît être très intéressante, cette découverte que le quotient d'un couple de noyaux est non seulement pratiquement indépendant de la grandeur nucléaire, mais encore remarquablement constant (d'une valeur moyenne de 0,82) dans tous les tissus que nous avons examinés. Il s'agit ici, à notre avis, d'une observation de portée biologique générale, d'un fait qui semble ne pas dépendre du type de tissu et de l'espèce animale (voir tableau III et fig. 3) pour autant que nous puissions inférer cette conclusion à partir de recherches faites sur des cultures *in vitro*. De telles données ne sont mentionnées nulle part dans la littérature que nous avons eu l'occasion de parcourir.

Comme la grandeur nucléaire diffère de manière caractéristique dans le tissu conjonctif de divers animaux (cf. (10, 11)), il est logique que la différence moyenne entre les deux partenaires nucléaires varie également dans ces tissus, tandis que le quotient ne le fait pas.

En ce qui concerne la taille nucléaire des cellules binucléées par rapport à celle des cellules à un noyau, les résultats des courbes de fréquences de grandeurs nucléaires ressortent des fig. 4 a et b. Dans les fibroblastes et les ostéoblastes du poulet, les noyaux d'une paire pris séparément ne sont pas plus grands que ceux des cellules uninucléées de la zone interne. En revanche, les fibroblastes des autres animaux que nous avons examinés (lapin, cobaye) et ceux de l'homme montrèrent un agrandissement des deux noyaux du couple d'environ $\sqrt{2}$ par opposition à ceux des cellules de la zone interne. Les sommes des volumes nucléaires dans les cellules binucléées de la zone externe se comportent de la même manière. Des recherches analogues sur des cultures de tissus ne nous sont pas connues. Cependant Ellenberger (16) trouva que les cellules hépatiques binucléées des mammifères contenaient fréquemment des noyaux plus petits que les cellules uninucléées. A son opinion se rallia également Münzer (29) qui ajouta pourtant que les noyaux des cellules binucléées, considérés chacun pour soi, étaient parfois plus grands que ceux des cellules uninucléées. Jacobj (21) et Clara (14) émirent l'hypothèse que les noyaux des cellules binucléées avaient en moyenne la même taille que ceux des cellules à un seul noyau; les noyaux du couple, pris ensemble, étaient aussi grands que ceux de classes nucléaires à volume double.

Cette observation concorderait avec nos résultats des cultures de tissus de poulet, pour autant que nous trouvions un volume nucléaire double dans la zone externe, comparaison faite avec le comportement *in vivo*. Si cependant nous prenions comme base la zone externe, dans laquelle nous avons effectivement trouvé nos cellules binucléées, on s'attendrait à voir le maximum de fréquences, correspondant au volume des deux noyaux additionnés, au niveau de K_4.

En examinant à nouveau le nombre des cellules binucléées, bien moins nombreuses dans la zone interne de nos cultures, nous avons pu voir que les noyaux montraient une grandeur moyenne correspondant à $K_{1/2}$ et que les sommes des volumes des deux noyaux s'accumulaient plus ou moins sur K_1, ce qui représente un comportement analogue à celui de la zone externe.

Nous nous sommes déjà demandés, à un autre endroit de notre publication, si le gain restreint en cellules binucléées dans la zone interne était conditionné uniquement par des raisons de méthode (à cause de la plus grande difficulté que l'on a de les reconnaître dans l'auréole de croissance plus dense à cet endroit) ou plutôt par des raisons biologiques. Maintenant, nous sommes probablement en droit d'admettre, sur la base de nos explications précédentes, que le besoin de former des cellules binucléées (2 fois $K_{1/2}$ à partir de K_1) y était généralement moins prononcé que dans la zone externe, où nous avons affaire à des noyaux de volume double et où l'augmentation relative de la surface paraît plus justifiée.

Nous ne pouvons pas expliquer pour le moment pourquoi nos cultures de fibroblastes de lapin, de cobaye et d'homme se comportent différemment de celles du poulet. Il nous paraît souhaitable que les résultats s'y rapportant soient contrôlés pour reconnaître si cette différence est causée par la nature biologique des tissus en question ou éventuellement uniquement par notre méthode de culture. Pourtant, Gauer (18) obtint un résultat semblable avec les cellules binucléées de l'épithélium de transition des voies urinaires, dont les noyaux (pris séparément) se comportent également, par rapport à ceux des cellules uninucléées de la couche basale, comme $\sqrt{2}$ à 1.

Habituellement, les cellules uninucléées de la zone externe croissent jusqu'à atteindre un volume double de celui des cellules de la zone interne; à ce moment intervient la division mitotique. Cependant, dans les cellules binucléées de nos cultures de tissu conjonctif de lapin, de cobaye et d'homme (cf. fig. 4 b), le volume du caryoplasme n'a pas doublé, mais augmenté de $2\sqrt{2}$, ou, pour s'exprimer d'une autre manière, la substance nucléaire a atteint une surface double: l'ordonnée qui correspond au milieu de la courbe se situe donc plus ou moins au milieu de la classe moyenne entre K_2 et K_4 (cf. Bucher et Gattiker (10)). Le fait que des noyaux pouvaient croître, dans des conditions particulières, de façon à doubler non leur volume mais leur surface, fut également mentionné par Hertwig (20) et déjà auparavant par Boveri (2) et Herbst (19). Il résulte de ce comportement que la surface nucléaire devient relativement plus petite par rapport au volume et que, par conséquent, les conditions des échanges entre caryoplasme et cytoplasme deviennent de plus en plus défavorables, point sur lequel de nombreux auteurs ont attiré l'attention à plusieurs reprises. Si l'on voulait donner une interprétation biologique aux observations citées précédemment, on pourrait imaginer que les noyaux cellulaires d'une grandeur de $2\sqrt{2} (= K_1 \cdot 2\sqrt{2})$ aient dépassé la taille optimum et que, par conséquent, il convienne d'agrandir la surface par division amitotique.

Quantitativement, le gain en surface dû à la répartition d'une masse nucléaire donnée sur deux noyaux, c'est-à-dire à la formation d'une cellule binucléée, se présente comme suit:

Prenons tout d'abord, en tant que corps géométrique le plus simple, un noyau sphérique de volume V_1 et demandons-nous comment augmente la surface si ce noyau se divise en deux noyaux sphériques de volume $V_{1/2}$; on obtient alors pour les surfaces le rapport de $2\ O_{1/2} : O_1 = 1{,}2597$. Afin de nous rapprocher davantage de la forme des noyaux de nos cultures, nous avons effectué les mêmes calculs pour un ellipsoïde de révolution, en employant la formule approximative de Legrand $O = 2\ b^2 + \frac{2\,a^2\,b\,\pi}{e}$ arc sin $\left(\frac{e}{a}\right)$ et en supposant que l'axe longitudinal b soit 2 fois aussi grand que a, comme c'est plus ou moins le cas pour nos noyaux. Dans de telles conditions, la somme des surfaces des deux noyaux $V_{1/2}$, résultat de la division d'un noyau V_1, se comporte par rapport à la surface de ce dernier comme 1,2599 à 1. L'augmentation de surface est ainsi pratiquement la même que dans le cas de la sphère.

Münzer (29, 30) et d'autres montrèrent que des cellules binucléées se rencontraient plus fréquemment non seulement lors d'exigences physiologiques accrues, mais encore lors de conditions de vie moins favorables. Cela concorde aussi avec l'observation de Levi (23) selon laquelle la fréquence des cellules bi- et plurinucléées augmente avec l'âge des cultures de tissus *in vitro*, c'est-à-dire avec l'altération des conditions de vie. L'apparition de cellules binucléées s'explique alors par des amitoses.

Ainsi nous avons déjà entamé le problème de la naissance des cellules binucléées (par amitose ou par mitose). Comme on admet presque généralement aujourd'hui que — certains cas particuliers d'expérimentation mis à part — l'amitose mène à la formation de cellules binucléées, nous ne voulons revenir sur ce problème que dans la mesure où nous pouvons y contribuer par nos propres recherches.

Nous avons déjà mentionné dans la division « Résultats de nos recherches » le pourcentage élevé de cellules binucléées dans des cultures de fibroblastes de lapin traitées à la trypaflavine ou à la colchicine. Nous n'avons plus qu'à rappeler ici que dans ces cultures l'augmentation des cellules binucléées est parallèle à celle des amitoses (17, 5, 9). La trypaflavine à la concentration de 1 : 600000, empêchant pour ainsi dire complètement l'activité mitotique (pour les détails cf. Bucher (4)), il est exclu, pour ces raisons déjà, que les cellules binucléées résultent d'une mitose sans clivage du corps cellulaire ou fusion secondaire du cytoplasme des jeunes cellules filles. En revanche, un noyau dont la caryocinèse est impossible peut faire appel, en désespoir de cause, à la division directe, qui est moins sensible aux influences nocives. Puisque, lors d'essais aussi bien avec la colchicine qu'avec la trypaflavine, les processus du métabolisme sont entravés sans autre par l'action du poison, l'agrandissement de la surface nucléaire, et de ce fait l'amélioration des échanges entre caryoplasme et cytoplasme, revêt une signification toute particulière. Les résultats de Barta (1) qui décrivit, également dans les cultures de tissus, un nombre plus élevé d'amitoses et de cellules plurinucléées en cas d'apport insuffisant d'oxygène, parlent dans le même sens. Cependant, dans du tissu conjonctif cultivé avec suffisamment d'extrait embryonnaire ou tissulaire et ayant bien poussé, les amitoses (à l'opposé des indications de Schachow (33) de même que les cellules binucléées (voir aussi notre tableau II) sont plutôt rares.

En faveur de la formation amitotique des cellules binucléées, également dans nos cultures de tissus normales, on peut alléguer que les deux noyaux d'une paire possèdent pour la plupart une grandeur inégale (cf. fig. 1 et 2).

D'après Pfuhl (32), une amitose n'entrerait en considération que si la masse totale du noyau en question correspondait au moins à celle d'un noyau tétraploïde, ce qui concorderait d'ailleurs avec nos résultats en ce sens que les noyaux de la zone externe de nos cultures atteignent ce volume, c'est-à-dire, comme nous l'avons mentionné parfois, doublent leur grandeur par rapport à celle des noyaux diploïdes *in vivo* comme ceux de la zone interne *in vitro*. Des résultats semblables furent obtenus par exemple par Jacobj avec du foie de souris et Clara avec du foie humain.

Münzer constata souvent, lors d'examens de cellules hépatiques binucléées, une colorabilité différente des deux noyaux, et Clara remarqua aussi parfois que l'un des deux noyaux était particulièrement dense comme d'ailleurs l'observèrent précédemment d'autres auteurs. Le deuxième noyau, plus ou moins pycnotique (de temps à autre) d'après Clara, aurait tendance, selon toute vraisemblance, à se dissoudre. Cette observation fut faite surtout sur des cellules à gros noyaux. Münzer, Stämmler (34) ainsi que Clara discutèrent, sur la base de pareilles altérations, la question de la transformation inverse de cellules binucléées en uninucléées (pour de plus amples informations, cf. les travaux cités). Nous ne pûmes faire de telles constatations sur les cellules binucléées de nos cultures de fibroblastes: les deux noyaux paraissent, comme il le fut déjà mentionné, effectivement équivalents; aucun signe d'une tendance à la dégénérescence de l'un des partenaires du couple nucléaire ne fut découvert. Nous n'avons pas fait d'autres observations qui nous auraient permis de tirer des conclusions sur le sort des cellules binucléées; c'est pour cette raison que nous ne discuterons pas cette question plus en détail.

RÉSUMÉ

Dans des cultures de fibroblastes et d'ostéoblastes de divers animaux et de l'homme, on trouve un pourcentage différent de cellules binucléées (cf. tableau II). Seul un quart environ des 407 paires nucléaires évaluées dans des cultures non influencées de tissu conjonctif ont la même taille, et ceci toujours dans les limites d'exactitude de notre méthode de mensuration. Pour tous les tissus examinés, le quotient moyen petit noyau sur grand noyau est pratiquement constant avec la valeur de 0,82; en outre, il est indépendant de la grandeur totale du couple nucléaire (cf. tableau III et fig. 3).

Dans nos cultures de fibroblastes et d'ostéoblastes de poulet, la taille nucléaire (somme des deux noyaux) des cellules binucléées concorde avec celle des cellules à un noyau de la zone correspondante (zone externe), tandis que, dans les cultures de tissu conjonctif de lapin, de cobaye et d'homme elle subit une augmentation du facteur $\sqrt{2}$ (cf. fig. 4).

En influençant expérimentalement nos cultures avec de la colchicine et de la trypaflavine en concentration appropriée, nous avons réussi à augmenter la fréquence des cellules binucléées qui, à notre avis, doivent leur origine à une division amitotique.

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CYTOLOGICAL OBSERVATIONS ON THE CYTOPLASMIC FACTOR "KAPPA" IN *PARAMECIUM AURELIA*

J. R. PREER, JR. and PEARL STARK[1]

Zoological Laboratory, University of Pennsylvania, Philadelphia, Pa., U.S.A.

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THE genetic cytoplasmic factor, kappa, is found in *Paramecium aurelia* and is associated with the "killer" trait (11). Animals containing kappa liberate a poison, paramecin, into the fluid in which they live, and are called "killers". Animals lacking kappa are killed by paramecin and are called "sensitives". Killers themselves are resistant to the toxic effects of paramecin. Preer (9) reported that kappa particles are DNA containing bodies, ovoid to rod-shaped, single or double, with singles varying from 0.2 to 0.8 micra in length. This report was based on the observation of particles in whole animals fixed in Schaudinn's and stained by the Feulgen technique or hydrolyzed and stained with Giemsa.

The present paper reports studies of kappa particles which have been freed from the whole animals, and observed both without fixation and after a variety of fixatives. It is found that the earlier method utilizing Schaudinn's, dehydration, and whole mounts gave a much distorted view of kappa. Using the same strains as in the first study, kappa is now shown to be much larger than previously reported, with single forms varying from 0.6 micron up to as large as 10 micra in length. Moreover, new details of internal structure have been seen.

MATERIAL AND METHODS

Stocks of *Paramecium aurelia* used in this study were the killers G, Gml, and 50 of variety 2 and 51 of variety 4; corresponding kappa-free sensitive strains of each were used. The origin of the stocks has been given (9). General methods of culture were those described by Sonneborn (12), except that the culture medium consisted of *Aerobacter cloacae* in a 0.75 per cent infusion of Cerophyl (Cerophyl Laboratories, Kansas City, Mo.) rather than *A. aerogenes* in baked lettuce infusion.

The initial observations, with phase contrast, of crushed stock G paramecia, showed the killers to have particles not found in the sensitives. Inasmuch as these particles were of sizes and shapes which might be confused with bacteria, it was

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essential to remove the bacteria in order that kappa be identified with complete validity. (It may be noted here that in stained whole mounts the bacteria are within vacuoles and kappa is in the cytoplasm, so that they are not likely to be confused.) This was accomplished by a technique similar to that described by Hetherington (5). Ten to twenty animals were introduced by means of a micropipette into one side of a spot plate depression containing sterile 0.25 per cent yeast extract. After the animals had migrated to the opposite side they were removed. Six successive migrations were made initially and then a series of three migrations was repeated every hour for four hours. The purpose of this procedure was to reduce the bacterial concentration to such an extent that bacteria were not likely to be observed by microscopical examination. Accomplishment of this purpose was demonstrated by washing sensitive strains as controls and examining them microscopically for bacteria by the procedures used for kappa examination. In all cases, these examinations revealed no bacteria. Transfer of single washed paramecia to agar slants and subsequent incubation usually showed no growth, and it was concluded that most individuals were completely freed of bacteria by the procedure.

Yeast were easily differentiated from kappa microscopically. It was, therefore, found expedient to establish bacteria-free cultures of killers and sensitives on a single strain of *Saccharomyces cerevisiae*, strain Mrak 93. Paramecia were cultured in 0.25 per cent yeast extract containing the living yeast. This was accomplished for all strains except 51 killer which appeared incapable of growing under these conditions.

Observations on unfixed kappa were made by crushing killers and observing the preparations microscopically. One to fifty paramecia were placed on a slide and as much fluid as possible removed with a micropipette without drying the preparation. When a cover glass was dropped on, the paramecia broke and the contents flowed out. The preparation was then sealed with vaseline and examined.

Fixation of kappa from broken paramecia was accomplished by placing about fifty animals on a slide and removing as much fluid as possible with a micropipette. Then a micropipette which had its tip fused was used to break up the paramecia. When most were broken up a liquid fixative was dropped on, or the slide was inverted over the opening of a bottle when vapor fixation was desired. Close examination under the microscope and considerable care had to be exercised when it was desired that the preparation should not become dried. Air dried preparations were obtained by allowing drying after the animals were broken with the fused pipette. In wet preparations it was found that kappa from some killer strains adhered to the slide much more readily than kappa from other strains. For example, the kappa from variety 2 of *P. aurelia* adhered very well while kappa from variety 4 was exceedingly difficult to stick to the slide. It was found that kappa stuck most readily when the preparations were virtually fluid free and contained at least 40 to 50 paramecia.

OBSERVATIONS

Fixed kappa in whole animals. Sketches of variety 2 stock G kappa in whole animals fixed in Schaudinn's and stained in Giemsa by previously described methods (9) are given in Fig. 1. Care has been taken to sketch only bodies which are oriented horizontally. Large numbers of kappa lie on end and the resulting optical section gives a picture of a somewhat smaller average kappa size than the picture obtained from the sketch. The size for singles was given by Preer (9) as about 0.4 micron long for stock G, but when orientation is considered, the average size of singles is estimated to be probably nearer 0.6 micron. Furthermore, three types of kappa bodies can be recognized: singles, doubles, and "brights". Singles (first four sketches in Fig. 1) are rod shaped. In doubles (fifth sketch in Fig. 1) the connection between the two portions is often not apparent, and it is difficult to exclude in any one instance the possibility that the double is two singles lying close together by chance. The large number of doubles and their distribution among the singles, however, makes this interpretation unlikely for most. "Brights" (last two sketches in Fig. 1) are broader than singles and doubles, and usually are longer than singles. They contain a central area which takes no stain at all. Since this region appears bright in unfixed material under bright phase (cf. next section), kappa particles with such regions are called brights. In most brights it is very difficult or impossible to see any connection between the two stained ends. The edge facing the middle in one or both of

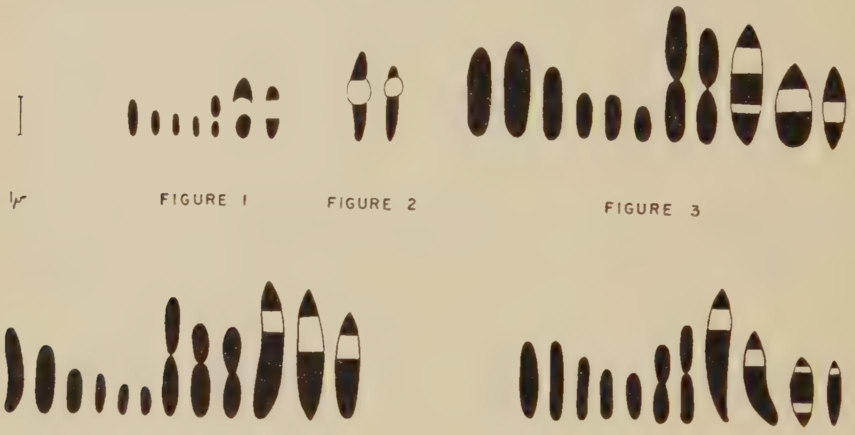

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

Figs. 1–5. Stock G kappa.
Fig. 1. Kappa in whole animals fixed in Scaudinn's, hydrolyzed in N HCl and stained with basic fuchsin. Fig. 2. Kappa in smears, fixed with Schaudinn's, observed with phase in water. Fig. 3. No fixation, animals broken and observed with bright phase. Fig. 4. Osmic fixation before breaking animals — aqueous medium, bright phase. Fig. 5. Osmic fixation after breaking animals — aqueuos medium, bright phase.

the two stained ends of the brights is often perfectly straight, and one of the ends is frequently smaller than the other. These brights in stock G are evidently similar to forms described earlier in stock 308-2, but not noted in stock G at that time. In fixed and dehydrated whole mounts brights are much more conspicuous in stock 308-2 than in stock G.

Unfixed kappa freed from the animals. Kappa of stock G observed immediately after animals are broken open presents a picture which is strikingly different from that of kappa in fixed whole animals. The bodies are much larger in the unfixed preparations. Sketches to scale of particles in a typical microscopic field under bright contrast phase are given in Fig. 3. If great care is used most of the details can be seen with an ordinary oil immersion microscope, but phase (either bright or dark) is of very great advantage. Typical singles are about one and a quarter micra long, but length varies from about one-half to three micra. They are quite uniform and no internal structure can be seen. Some doubles are long with but a slight constriction and others consist of two parts only barely connected; intermediate forms are also found. A careful examination suggests very strongly that the double and single forms represent stages in the multiplication of kappa by binary fission. Doubles, like singles, do not show internal structure. Brights appear with the central region dark in dark phase, ordinary oil immersion, and ultraviolet photographs; the region is light in bright phase. Brights are usually longer and broader than singles, and generally have pointed ends in contrast to the rounded ends of singles and doubles. Brights rarely appear double. When they do, one of the portions is usually typical while the other has the characteristic fusiform shape of a bright but lacks the refractile body. Typical forms are about two and a quarter micra, and exceptional individuals are four and a half micra long. No attempt has been made in the sketches to show the internal structure of the central region in the brights. Under phase, the region usually approximates a square, and frequently one sees that it consists of four rounded areas. Often the separation between the four areas is greater down the midline of the kappa particle and the areas are separated into two groups — one on each side of kappa. (In fixed and stained brights this material between lateral regions is sometimes stained and appears as a thin line connecting the two stained ends of the kappa particle.) Infrequently the refractile body is not square, but rectangular in shape, with its longitudinal axis perpendicular to the longitudinal axis of kappa. It is then seen as two parts. Occasionally kappas contain more than one bright region. Ultraviolet photographs show the two and four parted characteristics clearly (Fig. 9).

The presence of the bodies described as kappa above in the killer stocks and their absence in corresponding sensitive strains suggests strongly that they are in fact kappa. Conclusive evidence is provided by their resemblance to the stained particles in whole animals which have been shown to be kappa in previous studies. The particles from unfixed, crushed killers are sufficiently like the stained kappa particles in morphology that there can be no question concerning their identity. Furthermore, killers have been broken up, fixed in Schaudinn's and examined in water with phase (see Fig. 2). In spite of a considerable amount of shrinkage, the particles appeared much like unfixed particles. The position of particular brights, doubles, and singles was noted, and then the slide hydrolyzed, stained, and mounted just as the whole paramecia were treated. The appearance of individual particles became practically identical with particles in whole animals.

Kappa in unfixed whole animals. We see that fixed kappa in whole mounts differs considerably in size and details of structure from unfixed kappa of broken up killers. What is an unfixed kappa particle in an intact killer really like? The question cannot be answered directly, for it has not been possible to see such particles in intact animals by any technique. But it has been possible to obtain some information on the question indirectly as follows:

If the appearance of kappa in unfixed broken up animals does not represent the true condition of kappa in living animals, then it must undergo changes due to the change in the medium in which they are suspended. However, it is improbable that such changes occur, for when whole animals are fixed in the vapors of 2 per cent osmium tetroxide and then broken open and kappa examined with phase, it is found to appear a bit smaller but much more like unfixed kappa than kappa in Schaudinn-fixed whole mounts. Sketches of the particles in a typical field are given in Fig. 4. If the picture of kappa obtained by these two procedures (freeing particles from animals after fixation and without fixation) does not represent a fairly close approximation to the original state of kappa, then a rather unlikely assumption must be made, namely, that the distorsion of kappa produced by breaking open unfixed animals is similar to that produced by osmic fixation and subsequent breaking of animals. Not only is this explanation unlikely *per se*, but it is contrary to what is known about the action of osmic as a fixative. The character of the fixation obtained with osmic vapors was further shown by the fact that particles fixed after animals had been broken open (Fig. 5) appeared somewhat smaller than they did before fixation but not strikingly so. One gets from the drawings (and sometimes but not always from the original preparations) a distinct impression that unfixed particles, shown in Fig. 3, are larger than the fixed particles illustrated in Figs. 4 and 5. But when the actual mean lengths and widths are compared they do not show statistically significant differences within the limits of resolution of the microscope employed. For example, the mean difference in widths of the particles in each of the figures is less than 0.2 micron, and thus clearly not significant; differences in lengths are likewise not significant. We must conclude that the appearance of unfixed kappa from broken up animals is actually very close to its true state in the living animal.

The extent of the distortion produced by the procedure of Schaudinn's fixative, hydrolysis, staining, dehydration, and clearing is considerable. The typical form, about 1.2 micra long, shrinks to about 0.6 micron in length. Reduction in width is of comparable magnitude. The factor of $^1/_2$ for length becomes $^1/_8$ for volume, so that it appears that approximately $^7/_8$ of the volume of the kappa particle is lost by the procedure. It should be remembered that these estimates may be subject to considerable error because the optical systems used did not allow a resolution of better than 0.2 to 0.3 micron. At least two factors probably combine to cause this great distorsion. As already pointed out, Schaudinn's fixative causes a large amount of shrinkage, for kappa from broken up animals becomes much smaller after Schaudinn's. There also appears to be additional but less extreme shrinkage during dehydration and clearing.

The refractile body. The refractile bodies occurring in brights are sometimes found lying completely free of kappa in crushed animal preparations. The four-parted appearance of the "square" forms is highly characteristic and unlike any other structure observed in our cultures. Free bodies apparantly arise from the bursting of brights, for brights have been observed under phase to burst open and disappear in the medium, leaving only the intact refractile body. The rather rigid nature of the bodies is shown also by the fact that various fixatives, including Schaudinn's, cause a shrinkage of the kappa particle, but not the refractile body. Fixed brights then have a decided bulge in the center where the unchanged refractile body lies (Fig. 2). It should also be noted that refractile bodies show a considerable size range.

The refractile nature of the bodies is evidenced by their appearance in various media when observed by means of different optical systems. In an aqueous medium they appear light in bright phase, dark in dark phase, dark in ultraviolet photographs at 2537 and at 2750 Å, and dark with ordinary oil immersion. In glycerine or balsam or immersion oil they appear transparent and colorless with phase and with oil immersion. If the dark appearance in ultraviolet indicated absorption, one would expect high concentrations of nucleic acids. Consequently the bodies should stain deeply with basic dyes. The fact is that the bodies do not stain at all with basic dyes, indicating that the dark appearance in ultraviolet is probably due to refraction rather than absorption. This conclusion was confirmed at 2750 Å by photographs of kappa mounted in glycerine. The refractile bodies appeared transparent, while the rest of kappa still absorbed.

All attempts to discover the chemical nature of the refractile body by cytochemical procedures have failed. Negative results have been obtained with the McManus (8) technique for polysaccharides, Sudan stains for lipids (6), the Serra (10) test for arginine, and various basic dyes. The carbolfuchsin stain for bacterial spores is negative. In fact, it has not been possible to stain the refractile body with any stain. Possibly the bodies represent some simple inorganic compound; or perhaps they are covered with a thin layer of some very impermeable substance which does not in itself take up enough stain to become visible and does not allow the diffusion of dyes to the interior.

It was possible to control the proportion of kappa particles containing refractile bodies (brights) in stock 51 by controlling the rate at which the cultures of paramecia were fed. Paramecia fed for slower fission rates had a higher proportion of brights than paramecia fed for higher fission rates. In a typical experiment animals were subcultured from one culture into two test tubes, one fed daily for 0.1 fission per day and the other for maximum fission rate (about 5.0 fissions per day). After a week, animals were washed, crushed, and observed under phase. 100 kappa particles were counted in each of 10 animals at each fission rate. The number of brights out of the 100 observed for each of the 10 slowly multiplying animals was as follows: 15, 18, 35, 17, 20, 23, 21, 19, 14, 27. Corresponding counts for the rapidly multiplying cultures: 1, 1, 2, 2, 0, 0, 0, 2, 3, 0. The rapidly and slowly multiplying cultures were of the same antigenic constitution, both being about two-thirds serotype B, and one-third serotype D. (Cf. Sonneborn (13) for an account of serotypes in stock 51.) A difference in the life cycle was not completely ruled out in this experiment, for one-fourth of the slowly multiplying animals were in the 2-anlagen stage of autogamy while none of the rapidly multiplying animals were in autogamy. However, the consistency of the counts with no overlapping of numbers makes it unlikely that the difference in proportions of brights is due indirectly to life cycle differences of the paramecia.

It might be noted that in these experiments on the proportion of brights, an effort was made to ascertain the proportion of double kappa particles at low and high fission rates. A greater number of doubles was found at the higher fission rates in stock 51. However, due to the small magnitude of the difference, the subjective element involved in deciding whether to score borderline cases double or single, and the inability to confirm the observation on other killer strains, the result is not considered significant.

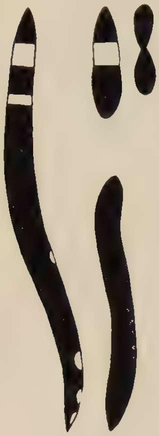

FIGURE 6

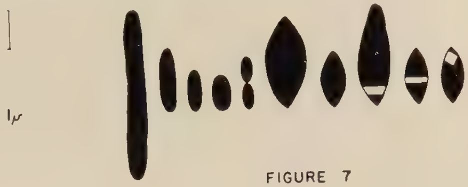

FIGURE 7

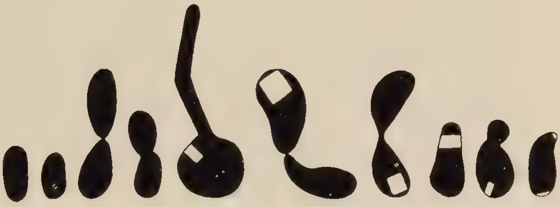

FIGURE 8

Figs. 6–8. Representative kappa particles from freshly crushed killers sketched under bright phase. Fig. 6. Stock 50. Fig. 7. Strain Gml. Fig. 8. Stock 51.

Kappa in different strains. The appearance of kappa in different killer strains in fixed whole animals has been described (9). The present method of observing kappa in crushed animals with phase is far superior to the previous method and more detailed and accurate comparisons can be made. The appearance of variety 2 stock G kappa under phase is described above, and sketches of random samples of particles appear in Figs. 3, 4, and 5.

Strain Gml is a mutant, probably of kappa, derived from stock G. It causes sensitives to become paralyzed before dying, rather than causing them to spin in the characteristic manner of stock G. Gml kappa was described earlier as being smaller than stock G kappa. Diagrams of representative Gml kappa particles appear in Fig. 7. Typical forms were chosen for illustration in the figure rather than a random sample. The average kappa size is possibly somewhat less that in stock G, but not noticeably so. The chief difference appears to be in a much reduced proportion of brights. In Gml grown at 0.3 fissions per day at 17° C with *Aerobacter cloacae,* counts of the

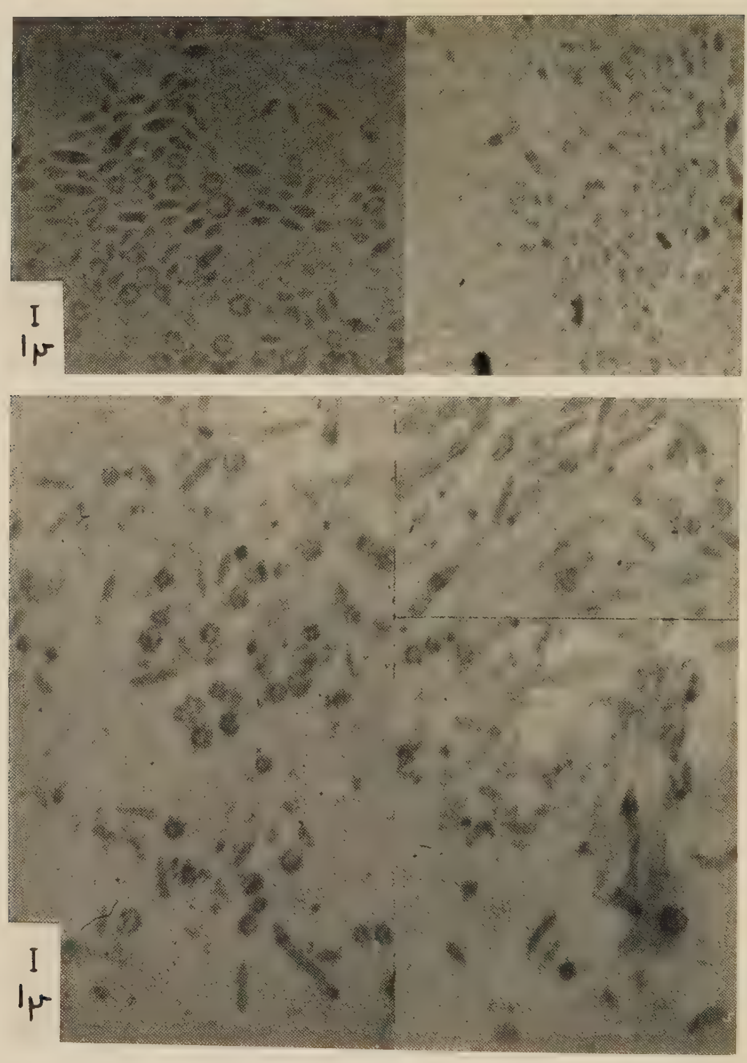

Fig. 9. Ultraviolet photographs of unfixed broken stock G killers. Upper photograph taken at 2750 Å and lower at 2537 Å. Elongated forms represent kappa. The refractile bodies appear dark and can be seen in many of the elongated particles.

number of brights among particles in each of five killer animals was 4, 6, 3, 4, and 4. Corresponding counts for stock G were 36, 21, 26, 27, and 19. Essentially the same proportions were found in the two stocks growing at 0.5 fissions per day at 27° C in bacteria-free cultures containing living yeast.

It was noted that in stock G brights are usually larger and have more pointed ends than singles. Rarely in stock G, singles lacking refractile bodies have this form. In stock Gml, large kappas with pointed ends, but lacking refractile bodies are much more common. It appears that the basic bright form (large size and pointed ends) is separable from the capacity to produce refractile bodies. The chief difference in G and Gml is apparently not in the proportion of particles with the bright form but in the capacity of these forms to produce refractile bodies. It was also observed that refractile bodies are usually smaller in Gml than in G.

Representative particles in variety 2 stock 50 (which kills in a way which is different from that of G and Gml (9)) are shown in Fig. 6. The extremely long forms (up to 10 micra) are not characteristic of this strain, having been observed on only one occasion. Their identity as kappa is unmistakable, however. Not only were all intermediate sizes observed, but the large forms contained the characteristic refractile bodies with the four- and two-parted appearance. Kappa in stock 50 is generally very similar to stock G kappa in morphology.

Kappa in variety 4, stock 51, which had been cultured at a slow fission rate (0.5 fission per day) is shown in Fig. 8. The general form is seen to be quite different from variety 2 kappa (G, Gml, and 50). The particles are much less slender, and brights do not have pointed ends as they do in variety 2. Brights are often double, with one part smaller than the other, and with the larger part invariably containing the refractile body. Particles frequently assume bizarre shapes, and many appear ''yeast-like'' in form. Forms like the fifth particle in Fig. 8 are seen only occasionally, while the other forms are much more common. It is interesting to note that the refractile bodies themselves are remarkably similar to those in variety 2. The four-parted appearance is identical in most stock 51 particles and in stock G; if anything it is more clearly seen in stock 51. Some of the refractile bodies in stock 51, however, appear rounded, and do not show subdivisions. When killers in stock 51 are allowed to grow at their maximum rate by supplying an excess of culture medium, the kappa particles appear smaller and uniform, consisting primarily of regularly shaped singles and doubles like the first four particles in Fig. 8; bizarre shapes and brights become much less frequent.

Cytochemical studies. Considerable effort was given to a study of the kappa particle using cytochemical procedures, but most of the results were negative. Negative tests were obtained for lipids (6), polysaccharides (8), cytochrome oxidase (in vital and fixed procedures using nadi reagent), and reducing enzymes (vital staining with neotetrazolium). A faint reaction which was suggestive, but unconvincing, was obtained for alkaline phosphatase (3). A considerable amount of shrinkage was encountered after all of the fixatives called for by these techniques. Because of the difficulty of ascertaining faint colors in such small objects, too much importance probably should not be attached to these negative results. Kappa is gram negative.

Kappa has already been shown to contain desoxyribonucleic acid (9). It was consequently not surprising to find a weak but definitely positive arginine test (10), suggesting that the DNA is associated with basic proteins.

An attempt was made to ascertain whether DNA is evenly distributed throughout the kappa particle, or whether it is localized into "nuclear" regions. Using acid hydrolysis followed by basic dyes such as Giemsa and basic fuchsin, it was sometimes possible to get differential staining of regions which was suggestive of nuclear structures. In all cases where differentiation was possible, smears had been previously air dried. In all material which had been fixed while wet and in which drying had been carefully prevented no differentiation was obtained. Hydrolysis with N HCl and with ribonuclease were equally ineffective. The techniques of De Lamater and Hunter (2) using thionin and azure A likewise gave no differentiation. Since differentiation with dried material was not obtained consistently and since kappa always appeared uniform in undried preparations, it is concluded that there is no good evidence at present for "nuclear" structures within kappa.

DISCUSSION

A striking and distinguishing feature of the kappa particles in all strains thus far investigated is the refractile body with its characteristic four- or two-parted appearance. It is interesting that the body is practically identical in such widely different kappas as those found in varieties 2 and 4 of *P. aurelia*. This is so despite the very different morphological appearance and physiological properties of the two. Perhaps the refractile body represents the accumulation of some metabolic product, or possibly it is a spore-like structure. Spores in bacteria, like the refractile body, are refractile, often appear under adverse nutritive conditions, and are difficult to stain. The possibility, however, seems unlikely in view of the fact that the refractile bodies are not stained by carbolfuchsin which ordinarily stains bacterial spores.

The detailed appearances of the double forms of kappa strongly suggest that kappa's mode of increase is by binary fission. Conclusive evidence of fission based on direct observation is not available.

The question of the nature of kappa has not been satisfactorily answered. Kappa was first detected by genetic methods and at that time was considered a part of the genetic system localized in the cytoplasm (11). Altenburg was first to point out that kappa may be an organism of extrinsic origin, suggesting initially that kappa is a "viroid" and later that kappa is a relative of the green algae of *P. bursaria* (1). Lindegren (7) has suggested that kappa is a symbiotic virus, and Preer (9) has compared kappa with Rickettsiae. Sonneborn (14) concluded that kappa appears intermediate between infectious viruses and normally present cytoplasmic elements of heredity. It would appear that one approach to a satisfactory solution to the question of the phylogenetic position of kappa is to obtain a thorough knowledge of its structure and physiology and then compare its attributes with those of possible relatives. Three points in the present paper are relevant to this discussion.

The first point is that in our experience most observers studying kappa in crushed animal preparations feel that in size and shape kappa resembles many bacteria more than it does viruses, algae, and various other forms. The protoplasmic constituents of kappa-free paramecia which we have observed do not have such shapes, with the possible exception of mitochondria, which characteristically appear as bent rods with swollen ends. However, in our opinion, kappa resembles many bacteria in shape and form more than it does mitochondria. Moreover, when unfixed paramecia are broken open by the techniques which we have described, the mitochondria, unlike kappa and bacteria, undergo a sequence of almost immediate changes in form, transforming from their normal shape to spheres. These changes appear to be similar to those observed in mammalian material (4).

The second and third points in this study which bear on the nature of kappa are negative and consist of the failure to get satisfactory evidence for the enzyme content of kappa and the failure, under favorable conditions, to demonstrate a "nucleus" and a "cytoplasm". It is felt that the inability to demonstrate "nuclear" regions is an important point of difference between kappa and bacteria, for the techniques which were used in this study have almost invariably shown differentiation within bacterial cells. The failure of all efforts to cultivate kappa *in vitro* also does not support the view that kappa is phylogenetically close to bacteria.

It should be remembered that certainly no one, or even several characters can be considered decisive in answering the question of the phylogenetic position of kappa; only by comparing the many attributes can a likely answer be obtained. Certainly on the basis of the facts brought out in this paper or even in considering all of our present knowledge about kappa a reliable conclusion as to kappa's nearest relative is not possible.

Finally, it should be pointed out that the recognition of kappa freed from paramecia makes possible a variety of new lines of inquiry. It should be possible to isolate kappa from paramecia and then to carry out macrochemical studies. Antigenic studies are possible using agglutination reactions. Attempts to cultivate kappa *in vitro* should be aided, due to the greater ease of identifying kappa, especially if cytological examination is combined with antigenic tests. Lastly, studies on the relation between the kappa particle and the paramecin particle should be facilitated. Preliminary work has been done on all of these problems and the results appear promising.

SUMMARY

The cytoplasmic factor kappa has been studied free from whole animals in several stocks of *Paramecium aurelia*. It was shown that no appreciable distortion occurred when kappa was freed from intact animals. However, considerable distortion was caused by the use of Schaudinn's fixative according to methods employed in earlier work. Kappa is rod-shaped, varying in length from 0.6 to 10 micra. Its form differs in different strains of killers. Kappa appears as singles, doubles (suggesting multiplication by binary fission), and enlarged forms containing a refractile body. The refractile structure usually consists of two or four parts of unknown composition and function. The proportion of forms with refractile bodies was shown to increase in one stock when the fission rate of the paramecia was reduced by a restriction of the food supply. Kappa reacts positively to the Serra test for arginine. Under phase kappa appears strikingly like bacteria. On the other hand, enzymatic content, "nuclear" differentiation, and *in vitro* growth could not be demonstrated.

The authors wish to express their appreciation to Dr. George Rudkin and Dr. Jack Schultz for the ultraviolet microscopy.

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THE ACTION OF PROTEOLYTIC ENZYMES ON THE VITELLINE MEMBRANE OF *PSAMMECHINUS MILIARIS* EGGS

A. MINGANTI

Wenner-Grens Institute, University of Stockholm, Sweden and Zoological Institute, University of Palermo, Italy

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In the sea urchin a close connection exists between the vitelline membrane of the oocyte, that of the mature egg, and the fertilization membrane. These structures, which derive from one another (13), reveal a different susceptibility to the proteolytic enzymes, thus making it possible to deduce some information about their chemical constitution and the changes they undergo during the processes of maturation and fertilization.

It is well-known that the vitelline membrane of the mature egg is very sensitive to the proteolytic enzymes. Observations on this subject are reported in the papers of Runnström, Monné and Broman (14), Runnström (12), Hultin (7), Runnström and Wicklund (19), Bohus Jensen (4, 5), Moore (9, 10). However, a detailed study as to the limits of such sensitivity is lacking. The present note concerns chiefly these quantitative aspects of the action of the trypsin, chymotrypsin and papain on the vitelline membrane of the mature egg of *Psammechinus miliaris*. As for the 'vitelline membrane' of the oocyte and the fertilization membrane, which in our experiments showed a remarkable resistance to the proteolytic action, the investigation is not yet complete and the results will be published later.

MATERIALS AND METHODS

The experiments were carried out at the Kristineberg Zoological Station, on eggs of *Psammechinus miliaris*, mostly of the Z-form.

Stock solutions, containing 0.1 per cent enzyme in sea water, were prepared of crystalline trypsin (General Biochemical), crystalline chymotrypsin (Armour), and papain (kindly supplied by Dr. T. Gustafson, who crystallized it from a crude preparation, according to the Balls and Lineweaver (2) method). The preparations of trypsin and chymotrypsin contained about 50 per cent salt whereas the papain preparation was practically salt free. More diluted solutions of trypsin and chymotrypsin were obtained by adding sea water (pH = 7.8, not corrected) to the stock solutions; the papain was activated by diluting its stock solution with a 0.03 M cysteine hydrochloride solution in sea water, freshly prepared (pH corrected to 7.8 by N NaOH).

The activity of the enzyme preparations has been tested by measuring their hemoglobin units according to Anson (1), and their viscosimetric units according to E. Hultin (6). The determinations were carried out at the Wenner-Grens Institute, Stockholm, by Dr. G. Lundblad, to whom I am greatly indebted. The data are reported in the Table I.

TABLE I

Solution in per cent	Hemoglobin units × 10^4	Viscosimetric units × 10^6
Trypsin 0.01	2.4	11.2
Chymotrypsin 0.01	1.2	2.1
Papain 0.01	2.2	4.4

The estimations of the activity were made with the enzymes dissolved in distilled water. The papain was activated: 1. by KCN according to Anson's method, for the determination of the hemoglobin units; 2. by 0.03 M cysteine hydrochloride, for Hultin's viscosimetric units.

The eggs were treated in the enzyme solutions at room temperature (about 22°C.). Afterwards they were washed, and the presence of the vitelline membrane was tested as described below. The jelly coat was not removed before the treatment.

OBSERVATIONS

Two tests for the presence of the vitelline membrane have been used: 1. the 'hypotonicity test'; 2. the 'fertilization test'.

1. For the hypotonicity test, as described by Runnström, Monné and Broman (14), the eggs are brought into a strongly hypotonic solution (75 per cent distilled water + 25 per cent sea water). In the normal eggs the vitelline membrane, after a short time, ruptures at one point, through which the cytoplasm flows out, but otherwise the membrane remains apparently intact for a while. On the contrary, if the vitelline membrane has been digested, it breaks down into granules, whereas the cytoplasm swells evenly, without flowing out. We observed that in this case too the breakdown of the vitelline membrane starts at one point, and proceeds very rapidly on the entire egg surface; the granules, for a while, are distinguishable from those of the cytolysed plasm, as they are coarser and arranged in one peripheral layer; but soon all granules mix together, owing to their Brownian movement.

The time necessary for the different concentrations of enzymes to dissolve the vitelline membrane of all eggs, as checked by the hypotonicity test, is reported in the Table II and in the graph of Fig. 1: the single data are the average of many determinations, which however gave rather close values. The trypsin and the chymotrypsin, at the same concentrations, showed the same activity.

TABLE II

Solution in per cent	Trypsin and chymotrypsin	Activated papain
0.001	2 minutes	1 minute
0.0003	10 »	—
0.0001	40 »	20 minutes
0.00003	1 hour 30 minutes	—
0.00001	3 hours 30 »	1 hour 30 minutes

2. For the fertilization test, the eggs, after the treatment, were inseminated with fresh sperm. The elevation of a fertilization membrane is assumed to indicate that the vitelline membrane is still present; if a fertilization membrane does not form, the vitelline membrane is supposed to have been digested.

In our experiments, a negative fertilization test always required a longer treatment than a negative hypotonicity test. That is, eggs treated for a short time, and whose vitelline membrane appeared as dissolved in a hypotonic solution, were still able to elevate a normal fertilization membrane upon insemination. Moreover, the fertilization test, as already observed by Runnström, Monné and Broman (14), demonstrates that the action of the proteolytic enzymes on the vitelline membrane proceeds by steps. The following example is given: eggs treated for 1 minute with a 0.001 per cent trypsin solution, reveal a dissolved vitelline membrane in a hypotonic solution; however, if inseminated, they elevate a normal fertilization membrane, which has a birefringence of the same value as the controls (retardation: 12 mμ). After a 2-minute treatment, a fertilization membrane can still form, but a closer and incomplete one (similar to the 'gelatinous rim' described by Runnström and Wicklund (19)), and having a weaker birefringence than the controls (retardation: 6 mμ). Lastly, after a 3-minute treatment, no fertilization membrane elevates any more; often the cortical granules are exuded as usual, constituting an adhering granular layer on the egg surface; such granules, during the cleavage, can be seen embedded in the hyaline layer (19).

The action of the trypsin has been tested on isolated vitelline membranes, obtained in the following manner: eggs were immersed into a hypotonic solution, so that the cytolysing plasm, flowing out, left the vitelline membranes almost empty. These membranes were washed in sea water and then transferred into a 0.002 per cent trypsin in sea water. For 1 hour no change was observed; after this period, the outlines of the vitelline membranes began to take a granular aspect. The digestion proceeded slowly, and only after 4 hours the vitelline membranes were completely broken down into coarse granules, carried away by Brownian movement. At the same time the controls (that is, empty vitelline membranes kept in sea water) were retaining their sharp outlines.

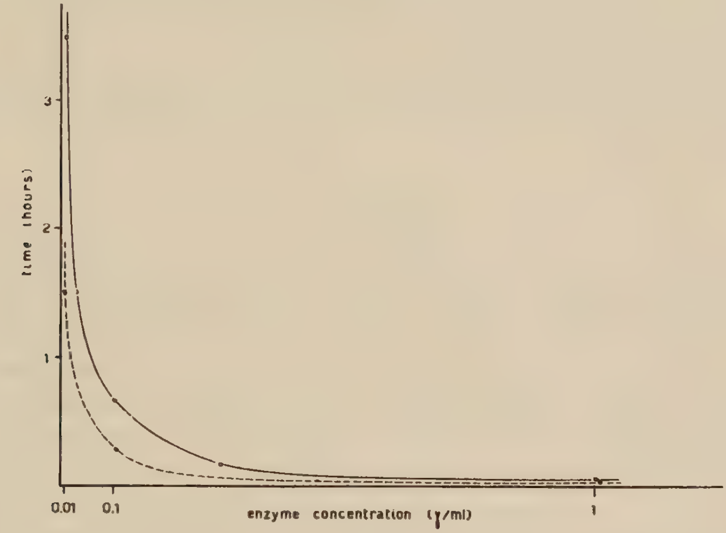

Fig. 1. Continuous line: trypsin and chymotrypsin. Dotted line: activated papain.

The breakdown of the vitelline membranes obtained in our experiments was actually due to an enzymatic action, as we tested by means of inhibitors. Eggs immersed in 1 ml of a 0.025 per cent trypsin solution added to 1 ml of 0.025 per cent Soya bean trypsin inhibitor, showed an intact vitelline membrane in a hypotonic solution, and elevated a normal fertilization membrane upon insemination, even when treated for 5 hours 30 minutes. This inhibitor has been ineffective on the chymotrypsin. As for the activators of the papain, the cysteine alone does not exert any splitting action on the vitelline membrane (14); on the contrary, eggs kept in a 0.03 M cysteine hydrochloride solution in sea water (pH brought to 7.8 by N NaOH) can elevate, upon insemination, a normal fertilization membrane at least 24 hours after their shedding, whereas the controls did not so 20 hours after the shedding.

The jelly coat too is dissolved by the proteolytic enzymes. The jelly coat was visualized by coagulating it in a 0.05 per cent crystalline lysozyme solution, followed by hypotonic treatment (16). Only relatively strong solutions of enzymes are effective. A 0.01 per cent solution of trypsin or chymotrypsin dissolves the jelly coat of all eggs within 2 hours; a 0.01 per cent solution of papain, activated with cysteine as described above, brings forth the dissolving in 15 minutes.

In our experiments we did not observe any activation of the *Psammechinus* eggs, owing to the enzymatic treatment.

DISCUSSION

1. The eggs of different species of sea urchins react differently to the proteolytic enzymes. Eggs of some species are activated by the trypsin, and elevate a fertilization membrane: that has been observed in *Echinocardium* by Runnström (12); in *Dendraster* by Moore (9, 10); by ourselves in *Brissopsis lyrifera*. Such activating effect, on these irregular sea urchins, occurs even with crystalline trypsin (9, 12). In *Psammechinus*, Runnström, Monné and Wicklund (15) reported a nuclear activation: that is to be attributed to impurities in the enzyme preparation, because, as already noted by Runnström (14) and Bohus Jensen (3), the crystalline trypsin does not bring forth any activation.

This different reaction to the trypsin might be related to a different chemical constitution of the vitelline membrane in the various species of sea urchins. Unfortunately, no comparative data are available on this subject.

2. As for *Psammechinus miliaris*, little is known about the chemical constitution of its vitelline membrane. In a recent paper, Lansing and Rosenthal (8) report that the vitelline membrane of *Arbacia* exhibits a marked basophilia with toluidine blue; the basophilia is greatly reduced after treatment with ribonuclease *in vivo*, and then the vitelline membrane appears strongly metachromatic. Anyway, the extreme sensitivity of the vitelline membrane of *Psammechinus* to the proteolytic enzymes clearly demonstrates that the backbone of its structure is formed by polypeptide molecules.

The most active enzyme is the activated papain; among the other enzymes, the chymotrypsin appears to be more active than the trypsin (cfr. Tables I and II). The curves of the Fig. 1 are, with a fair approximation, equilateral hyperboles; in their equations (time × concentration = a constant), the ratio between the constant relative to the papain and that of trypsin and chymotrypsin is about 0.5; that is, roughly speaking, the activity of the papain is two times higher than that of the trypsin and chymotrypsin (disregarding the corrections due to the different standard units of the enzyme preparations used). The higher activity of the papain is likely to be due to its lesser specificity. As a matter of fact but little is known about the specific action of the papain; whereas it is known that the chymotrypsin requires for its action the presence of an aromatic ring formed by tyrosine or phenylalanine residues, and the trypsin acts on linkages involving arginine or lysine (20). A similar case of higher activity of the papain than the trypsin, concerning the hyaline layer of fertilized *Arbacia* eggs, has been reported by Northrop (11).

The breakdown of the vitelline membrane, brought forth by the proteolytic enzymes, is not so fast as it appears at the hypotonicity test. We suppose that in this test the dissolving of the membrane into granules is favoured by a pressure exerted by the cytolysing egg plasm. This does, however, not explain why the dissolution of the empty vitelline membranes takes a much longer time. The vitelline membrane has here been exposed to exudates from the cortex, these being released in connection with the cytolysis of the egg in the hypotonic medium. The membrane is not only more resistant against proteolytic attack, but in contradistinction to the vitelline membrane *in situ* it exhibits a measurable retardation (6 mμ).

As described above, eggs which in hypotonic solution exhibit a dissolved vitelline membrane, sometimes (after a short enzymatic treatment) do elevate a normal fertilization membrane. Thus, we could assume that in the vitelline membrane there are peptide links which are broken very easily by the proteolytic enzymes; other links, on the contrary, resist longer, and upon insemination can be consolidated by cortical material, as usual, to form a fertilization membrane. This view seems to be supported by the peculiar fertilization membrane which we observed when eggs were treated with a 0.001 per cent trypsin solution for 2 minutes, and then fertilized and transferred (45 minutes after the fertilization) in a 0.01 per cent solution of Duponol (about this detergent, cfr. Runnström, Tiselius and Lindvall (17)). These membranes appeared to be constituted by a network of birefringent fibres closely interwoven, and leaving a few larger holes (Fig. 2); it is tempting to consider such a structure as a tougher frame, being present even in the normal fertilization membrane.

The above observations on the vitelline membrane of the mature egg have to be supplemented by the results of the experiments, which are being carried out, on the action of the proteolytic enzymes on the vitelline membrane of the oocyte and on the fertilization membrane.

3. The susceptibility to the proteolytic enzymes of the jelly coat, already pointed out by Runnström, Tiselius and Vasseur (18), must find its explanation in the chemical structure of this substance. The jelly coat is a mucin, thus being constituted by a proteic moiety and a carbohydrate one. The former contains, at least in *Echinus, Paracentrotus* and *Strongylocentrotus droebrachiensis,* lysine, arginine, phenylalanine and tyrosine, together with other amino acids (23); as for *Psammechinus,* no analytical data are available, but at least some of the mentioned amino acids must be present, owing to the specific action of the trypsin and chymotrypsin. According to Tyler (22), the protein and carbohydrate constituents do not exist as such in the molecule of the jelly coat, but rather the various sugars and amino acids are interlinked. On the contrary, for Vasseur (24) there is evidence that the carbohydrate portion is a polysaccharide, and the amino acid containing part is a protein; both are firmly linked to each other, probably by glycosylaminic links. From our results it could only be deduced that peptide links, involving lysine and/or arginine, and aromatic amino acids, are likely to be present in the backbone of the jelly molecule.

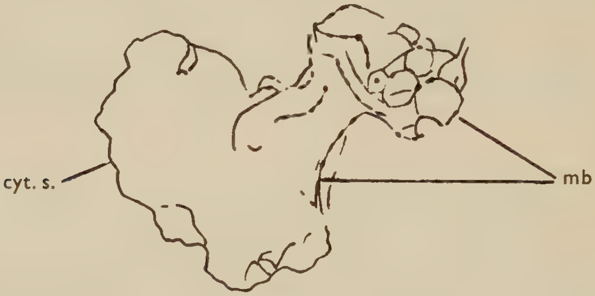

Fig. 2.

For the jelly, as well as for the vitelline membrane, the papain is more active than the other enzymes, probably owing here too to a lesser specificity of the papain. The jelly requires, for its dissolving, a higher concentration of enzymes than the vitelline membrane, and a much longer period of treatment. Such resistance to the proteolytic action has been reported for the mucin constituting the jelly of an amphibian egg (*Triturus*), which requires a strong solution of papain (1–3 per cent) to dissolve it, acting for 6–10 hours (21).

SUMMARY

1. The vitelline membrane of the mature egg of *Psammechinus miliaris* is digested very easily by the proteolytic enzymes. Quantitative data are given on this subject: a 0.001 per cent solution of trypsin or chymotrypsin dissolves the vitelline membrane within 2 minutes; a 0.00001 per cent solution, within 3 hours 30 minutes. The times relative to the activated papain are respectively 1 minute and 1 hour 30 minutes.

2. The jelly coat too is dissolved by the proteolytic enzymes. A 0.01 per cent solution of trypsin or chymotrypsin takes 2 hours; a 0.01 per cent solution of activated papain takes 15 minutes.

3. The chemical constitution of the vitelline membrane and of the jelly coat is discussed on the basis of the experimental results.

I am greatly indebted to the Swedish Institute, which rendered my stay in Sweden possible. I wish to express my gratitude to Prof. John Runnström, for suggesting this investigation and for all his help and guidance. My sincere thanks are also due to the authorities of the Kristineberg Zoological Station, for their great kindness and generous working facilities.

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MITOTIC ACTIVITY AND GROWTH FACTORS IN THE LIVER OF THE WHITE RAT[1]

H. TEIR and K. RAVANTI

The Institute of Forensic Medicine, University of Helsinki, Finland

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The liver of adult mammals is a "stable" organ in which mitoses are rare after the end of the growth period. In the liver of the white rat, mitoses have been found during the first eight weeks of life (15, 22). In the liver of the adult rat, mitoses are extremely rare (9, 15, 22). Brues and Marble (1) found that 0.005 per cent of the liver cells are in mitosis.

The purpose of this study has been to investigate the normal mitotic ratio in the liver of Wistar rats with regard to the effect of colchicine and tissue suspensions.

METHODS

The experimental animals all received the same treatment. The percentage composition of the food was: Wheat bran, oatmeal, rye-flour and oat bran 22.2 each, casein 4.3, butter 2.2, yeast 2.2, calcium lactate 1.0, salt 0.7, and cod-liver oil. After sacrifice of the animal pieces of the median lobe were immediately fixed in Bouin's solution. The 5 μ thick sections were stained with hemalum-eosin. With binocular 10 and objective 40 the mitoses were counted in 200 fields of vision chosen at random. The number of mitoses per 100 fields of vision was used as an index of the mitotic ratio.

To test different tissue suspensions 2- to 3-month-old animals were mostly used, since at this age the liver of the rat has the typical adult appearance (15), while mitoses are hardly seen in the organ any longer (15, 22).

OBSERVATIONS

Normal mitotic activity

In order to determine the mitotic ratio in normal liver tissue 13 different age groups from 2-week-old embryos to 12-month-old animals were used. In the liver of embryos and newborn rats fairly large numbers of the hematopoietic cells were found, which often divided mitotically. To a certain extent these mitoses rendered counting of the mitoses in the liver cells difficult. The mitotic ratio in the different age groups was as follows:

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[1] This investigation was made under a grant from the Sigrid Jusélius Foundation.

2-week-old embryos	15, 18, 37
Newborn rats	11, 16, 13
1-week-old rats	16, 12, 10
2- »	10, 12, 10
3- »	8, 12, 10
4- »	4, 2, 4
6- »	2, 3, 3
8-week-old rats	2, 1, 1
10- »	0, 0, 0
12- »	0, 0, 0
5-month-old rats	0, 0, 0
7- »	0, 0, 0
12- »	0, 0.

The mitotic ratio within the same age group was independent of the body weight. The mitotic values above and similar tables later are arranged so that the first figure concerns the animal with the lowest and the last that with the highest body weight. The mitotic ratio was independent of the animal's sex.

Colchicine sensitivity

Colchicine was administered subcutaneously into the interscapular region of both young and old rats.

2-month-old rats. Six rats received 0.2 mg colchicine. The time of influence was 10, 27, 54, and 72 hours. Mitotic values:

10 hours 18, 27 hours 2,0, 54 hours 13,8, 72 hours 4.

3-month-old rats. Three rats received 0.2 mg colchicine. The mitotic values were 0 and 0 after 24 hours and 33 after 47 hours.

3 $^1/_2$-month-old rats. Four rats received 0.02 mg colchicine. All were decapitated 22 hours later. In three animals the mitotic value was 0 and in one 2.

11-month-old rats. The group comprised 23 rats. Three of these received saline solution and 20 received 0.3 mg colchicine. The mitotic values following the colchicine injection were as follows:

Saline solution	24 hours	0, 0, 0
Colchicine	2 »	0, 0, 0
»	5 »	2, 2, 0
»	8 »	0, 0, 0
Colchicine	12 hours	0, 2, 0
»	18 »	0, 4, 0
»	24 »	1, 0, 4
»	36 »	0, 0.

No relation between mitotic values and body weight could be observed.

Homologous tissue supensions

A marked stimulation of the mitotic cell division in homologous organs has been obtained by injecting small quantities of mashed tissue. This has so far been achieved in the outer orbital gland, which is a stabile organ (23, 24) and in the epidermis (26) and the epithelium of the stomach of the rat (29), which are labile organs.

Immediately after decapitation of the rats a fixed quantity of liver was weighed and crushed in a porcelain mortar together with a small quantity of saline solution. Saline solution was then added so that different concentrations were obtained. Thus 1 ml of tissue suspension always corresponded to a certain quantity of liver tissue. The suspensions were injected intraperitoneally immediately after they had been prepared.

Apart from the liver, the mitotic ratio was also determined in the paired outer and inner orbital glands, which consist of similar tissue. Just as in the liver, mitosis is a rare phenomenon in these glands after the growth period (21, 22).

Liver suspensions from 2-week-old rats. The effect of the suspension on mitosis was simultaneously tested on 2- and 12-month-old rats. The quantity varied, while the time of influence remained at 48 hours. Mitotic values:

	Liver		Outer orbital gland		Inner orbital gland	
	2 months	12 months	2 months	12 months	2 months	12 months
0.02 mg	13	2	0	1	0	1
0.1 »	5	6	0	1	0	1
0.4 »	3	2	0	0	0	0
3 »	5	31	0	0	0	0
24 »	10	9	0	0	0	0
188 »	8	12	0	1	0	0

Mitoses were thus found in the liver of all animals and they were as frequent in 12-month-old rats as in the 2-month-old ones. There was, however, hardly any activation of the mitotic ratio in the orbital glands.

Liver suspensions from 12-month-old rats. This suspension, too, was simultaneously tested on 2- and 12-month-old rats. The time of influence was again 48 hours. The doses were the same as in the previous experiment. Mitotic values:

	Liver		Outer orbital gland		Inner orbital gland	
	2 months	12 months	2 months	12 months	2 months	12 months
0.02 mg	0	0	0	0	0	0
0.1 »	1	0	0	0	0	0
0.4 »	0	2	0	0	0	1
3 »	0	0	0	0	0	0
24 »	—	2	0	0	0	0
188 »	0	0	0	0	0	0

The effect was thus considerably smaller than that obtained with liver suspensions from 2-week-old rats.

Heated liver suspensions. Since the mitosis-stimulating factor in both the orbital gland (24) and the epithelium of the stomach (20) has proved thermostable, an experiment was made on 14 7-month-old rats, which received a liver suspension, heated for 15 minutes, from 2-week-old rats. The following temperatures were used: 20°, 30°, 60°, and 100° C. The quantity, 350 mg, was the same for all animals. The mitotic ratio in the liver (L), in the outer orbital gland (0) and in the inner orbital gland (I) was:

	20° C			30° C			60° C			100° C		
	L	O	I	L	O	I	L	O	I	L	O	I
4 hours	29	0	1	4	1	0	0	0	0	0	—	1
20 »	2	2	1	5	1	0	0	0	0	0	0	0
48 »	1	0	0	13	0	1	0	0	1	0	1	0
72 »							0	0	0	1	1	1

The experiments indicate that the mitosis-stimulating factor in liver suspensions is rather thermolabile. The heated liver suspension did not influence the mitotic ratio in the orbital gland any more than did the fresh suspension.

Heterologous tissue suspensions. As heterologous tissue the outer orbital gland of 12-day-old rats was used. The suspensions were obtained in the same way as the liver suspensions. They were tested on 2- and 12-month-old rats. The time of influence was 48 hours and the doses were 94, 47, 12, 3, 0.75, 0.4 and 0.2 mg of tissue. With these doses the corresponding mitotic values in the liver of 2-month-old rats were 0, 2, 0, 4, 4, 2 and 1 and of 12-month-old rats 0.2, 0, 0, 0, 0 and 0. The younger animals thus showed a slight mitotic reaction.

In another experiment 12 mg of heated orbital gland suspension were injected into 7-month-old rats. The suspensions were heated: 15 minutes at 20°, 60° and 100° C. The time of influence was 4, 20 and 48 hours. The mitotic values were as follows:

	20° C	60° C	100° C
4 hours	3	0	0
20 »	1	0	0
48 »	4	5	4

In all groups the mitotic value was greatest 48 hours after the injection. The heat treatment did not decrease the mitotic ratio in the liver in the same way as it did with the liver suspension.

Influence of colchicine and cortisone on the mitosis-stimulating effect of homologous tissue suspensions

A group of 36 4-month-old rats were used for this experiment. As tissue suspension 3 mg of the liver of 2-week-old rats was used. The 0.2 mg dose of colchicine was given subcutaneously. The 10 mg dose of cortisone was given intraperitoneally 4 hours before decapitation. The time of influence of the tissue suspension and the colchicine was again 48 hours. Each experimental group comprised 4 to 5 animals. The mitotic ratio for the different experiments was:

Liver suspension 4 hours	2, 4, 0, 2
» » 24 »	0, 14, 5, 4
» » 48 »	0, 3, 6, 6
Colchicine	3, 27, 41, 13, 9
Colchicine + 15 minutes ether anesthesia	20, 14, 16, 33, 6
Liver suspension + colchicine	14, 2, 15, 8, 9
Liver suspension + cortisone	9, 10, 5, 21, 11
Saline solution, 24 hours	0
» » 48 »	0, 0, 0

With liver suspensions a clear stimulation of the mitotic activity was again achieved. The mitotic ratio obtained with cortisone was high as well. Colchicine and cortisone do not seem to influence the mitotic activity of the suspensions to any noticeable degree.

DISCUSSION

The mitotic ratio in the liver of normal Wistar rats was approximately the same as in similar investigations made previously (15, 22). In newborn rats we found a higher mitotic ratio than that established by McKellar (15), although we tried to exclude mitoses in hematopoietic cells, principally normoblasts, and in reticular cells. On the other hand the decrease in the mitotic ratio at the age of 4 weeks corresponds well with previous investigations (15, 22), as does the rare occurrence of mitoses in liver of adult animals (9, 15, 22).

With colchicine, mitoses blocked in metaphase were also obtained in adult animals, which confirms previous results (8, 11, 16, 22). With colchicine administered perorally Kantner (10) found colchicine-mitoses in the liver of adult rats. These facts argue in favour of the correctness of earlier opinions that colchicine has both a mitosis-inhibiting and a mitosis-stimulating effect (17, 21). The effect of the colchicine was not more influenced by anesthesia than in previous investigations (11, 22).

The experiments with tissue suspensions are of particular interest. With varying doses of a suspension of 2-week-old liver a clear stimulation of the mitotic activity was obtained in 2-, 4-, and 12-month-old rats. With a suspension of 12-month-old liver the effect was insignificant even though the experiments were performed in exactly the same way. A similar age difference was observed between tissue suspensions of the outer orbital gland (23).

Marshak and Byron's (12) observations also argue in favour of an age-conditioned growth factor in the liver. By studying the effect of age on mitosis they found that the interval between partial hepatectomy and the maximum mitotic count increased directly with the age of the animal. The difference in the effect of the suspensions of 2- and 12-month-old liver corresponds well with Glinos and Bartlett's (6) investigations on the growth potential of normal rat liver. By means of the tissue culture method they found that the growth potential was an inversely proportional function of the age of the animals.

The mitotic stimulation by tissue suspensions indicates that in mashing liver tissue a mitosis-stimulating agent becomes activated. Marshak and Walker (13) succeeded in fractioning two different fractions out of chromatin, one of which had a mitosis-stimulating and the other a mitosis-inhibiting effect on the mitoses in hepatectomized rats after intravenous injection. The latent period was here only 3 hours. In our experiments stimulation of mitotic activity was also observed 4 hours after application of the tissue suspension. With large quantities of tissue suspension an increased mitotic activity was also obtained in the liver (14, 28), generally 6 to 7 days after application. It is possible, however, that this is a secondary regeneration phenomenon as a result of the injury caused by a larger quantity. After such an injection degenerative nuclear changes have been observed in almost half of all the liver cells (14).

In the liver of parabiotic rats Christensen and Jacobsen (5) observed a clear increase of the number of mitoses in both animals after hepatectomy on one of the parabions.

The mitoses induced by the tissue suspensions were morphologically quite normal. Determination of the different mitotic stages showed that the distribution was approximately the same as in normal young rats and in suspension-treated adult rats. A similar result was obtained when the distribution of the different mitotic stages in the outer orbital gland was investigated after injection of orbital gland suspension (23).

The mitosis-stimulating agent in mashed liver tissue seemed to be fairly thermolabile in contrast to the corresponding agent in the orbital gland (24) and in the epithelium of the stomach (20) of the white rat, both of which are thermostable.

The liver suspensions do not affect the mitotic ratio in the outer and inner orbital glands. On the other hand a suspension of outer orbital gland had a slight mitosis-stimulating effect upon 2-month-old rats and almost none upon 12-month-old ones.

Thus there seems to be a certain specificity which, however, does not follow the same rules as in the orbital gland and the mucosa of the stomach of the white rat. The fact that the effect margin in this respect is greater in the liver corresponds well with the observation that there is a greater effect margin in the liver with regard to sensitivity to tissue ribonuclease as compared with the orbital gland (27).

The increased mitotic activity in the outer orbital gland produced by a homologous tissue suspension could be influenced by simultaneous application of colchicine (23). In our experiments the mitotic value was somewhat higher in series which both received a liver suspension and colchicine than in animals which were given liver suspension alone, but it was not quite as high as in animals which received colchicine alone.

Cortisone has not as high an antimitotic effect on the liver as it has on the epidermis (19, 25); in the latter organ, shock (2, 3), and stress (4, 7) also produce a clear mitotic depression. A similar passivity to cortisone as regards mitotic activity has been demonstrated in the small intestine and in the proliferation obtained with thio-uracil in the thyroid gland (10) and in the outer orbital gland (25). No general antimitotic effect can therefore be attributed to cortisone.

SUMMARY

In the liver of the white rat mitoses occur in greater number during the first three weeks of life, and solitary mitoses occur up to the age of 8 weeks, after which mitoses are hardly seen in the organ any longer. The colchicine sensitivity is greater in young than in old rats.

Suspensions of liver tissue injected intraperitoneally clearly stimulate the mitotic ratio in the liver. With suspensions from younger animals the effect is considerably greater than with suspensions from old animals. The mitosis-stimulating agent seems to be rather thermolabile. Liver suspensions did not produce any stimulation of the mitotic activity in the outer and inner orbital glands. A suspension of outer orbital gland, however, gave a slight stimultion of the mitotic activity in the liver. The mitosis-stimulating effect of the liver suspension was hardly influenced by a simultaneous application of colchicine and cortisone.

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ALTERATIONS OF THE INORGANIC PHOSPHATE AND ARGININE PHOSPHATE CONTENT IN SEA URCHIN EGGS FOLLOWING FERTILIZATION[1]

E. L. CHAMBERS[2] and T. J. MENDE[3]

Department of Biology, New York University, New York City; Department of Anatomy, Johns Hopkins University School of Medicine, Baltimore, Maryland, and Lilly Research Laboratories, Marine Biological Laboratory, Woods Hole, Mass., U.S.A.

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Runnström (23), Zielinski (26) and Örström and Lindberg (22) measured the quantity of inorganic and organic phosphates in the acid soluble extracts prepared from the eggs of the sea urchin *Paracentrotus lividus.* Following fertilization, no changes could be detected. Chambers and White (6), however, found that after fertilization of *Strongylocentrotus purpuratus* eggs a prominent decrease in the quantity of inorganic phosphate occurred in the acid soluble extracts. This was accompanied by a corresponding increase in the quantity of phosphate which could be liberated from organic combination by hydrolyzing the egg extracts for 7 minutes in 1 N HCl at 100° C. White and Chambers (25) and Chambers and Mende (5) specifically identified and measured the quantity of adenosine triphosphate in several different species of echinoderm eggs. No change was found in the concentration of adenosine triphosphate in starfish eggs before and after insemination. Subsequently, Mende and Chambers (19) found arginine phosphate in the eggs of *Strongylocentrotus dröbachiensis* and *Asterias forbesii.* In order to determine whether the quantity of this compound changes following fertilization, the experiments performed by Chambers and White (6) were repeated, using the eggs of *S. dröbachiensis.* The results are presented in this paper.

The eggs of the two species of *Strongylocentrotus* mentioned are particularly suitable for studies of this kind, since, by virtue of the extraordinarily low inorganic phosphate content, minute variations in this and in the quantity of P liberated after hydrolysis may be measured with considerable accuracy. Previous investigators (23, 26, 22), who did not have the advantage of using immediately related species, worked with the eggs of *Paracentrotus lividus*, which possess a high inorganic phosphate content. As pointed out earlier (19), in eggs of species with a high inorganic phosphate content, complete barium fractionation is necessary for the demonstration of changes in the easily hydrolyzable phosphate compounds of the magnitude to be expected.

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[2] Now at University of Oregon Medical School, Portland 1, Oregon.

[3] Now at Lobund Institute, University of Notre Dame, Indiana.

During the course of the investigation, in order to avoid the breakdown of easily hydrolyzable phosphate compounds, it was found essential to handle the eggs with extreme care, and to use dilute egg suspensions at concentrations less than 2 ml of eggs per 100 ml of sea water.

METHODS

A dilute suspension of *Strongylocentrotus dröbachiensis* eggs was prepared and volume determinations made as previously described (5). Duplicate samples were removed at intervals of time before and after insemination, treated with 5 per cent trichloroacetic acid at 0° C, and the trichloroacetic acid soluble extracts prepared (5). The period which elapsed from the moment of adding trichloroacetic acid to the time of neutralization was maintained constant at 50 minutes. The neutralized samples were kept frozen at —20° C until all extracts had been prepared. They were then thawed and analyses for inorganic phosphate using the previously described method (5) were made simultaneously in all samples. Subsequently, analyses for Δ 1 min.[1], P and Δ 7 mins.[2]. P were carried out on the unfractionated extracts using the same method. Total phosphorus determinations were performed using the method of King (11) and Fiske and Subba Row (8), and reducing sugars were measured by the method of Folin and Malmros (9). Protocols of the three experiments described in this paper are presented below.

Experiment 1. A quantity of eggs from several females was suspended in sea water in a beaker kept at 14.7° C. The concentration of eggs in the suspension was determined as follows: While the contents of the beaker were being well stirred with a glass rod, 10 ml were removed using a transfer pipette with a wide (0.7 cm) mouth, the eggs washed once with a mixture of 95 parts 0.53 M KCl and 5 parts 0.52 M NaCl, pH 6.0, to remove the jelly, resuspended in sea water, centrifuged at 2000 × *g* for 10 minutes and the volume of packed eggs measured. This gave a value of 0.96 ml of eggs per 100 ml of suspension. Throughout the experiment the eggs were kept in suspension by means of a slowly rotating glass stirrer. For chemical analysis, the first sample, 100 ml, was withdrawn, centrifuged by hand at 100 × g for 3 minutes, the supernatant sea water discarded and an equal volume of 10 per cent trichloroacetic acid added 93 minutes after the eggs had been removed from the ovaries and 98 minutes before fertilization, as shown in Table I.

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[1] Δ 1 min. P represents the total inorganic P after 1 minute's hydrolysis in 0.1 N trichloroacetic acid at 100° C, minus the inorganic P content before hydrolysis. Under these conditions arginine and creatine phosphates are completely hydrolyzed to the free amino acids and inorganic phosphate (15); 4 to 5 per cent of the labile P of adenosine pyrophosphate is liberated (15) and 55 per cent of the P of glucose-1-phosphate is set free (19).

[2] Δ 7 mins. P represents the total inorganic P after 7 minute's hydrolysis in 1 nN HCl at 100° C, minus the inorganic P content before hydrolysis.

The insemination of the eggs in the beaker was performed by rapidly dispersing a small drop of freshly obtained "solid" sperm throughout the contents of the beaker. Several drops of the suspension were examined and it was found that fertilization membranes had formed and were highly elevated within 2 minutes in all the eggs. Cleavage occurred normally and over 95 per cent developed into healthy swimming blastulae.

Experiment 2. Eggs collected as in Experiment 1 were kept at 14.5° C and the suspension concentration was 1.66 ml of eggs per 100 ml suspension. The first sample for chemical analysis was taken 60 minutes after removal of the eggs from the ovaries and 31 minutes before fertilization (Table I). These, when inseminated, developed as in Experiment 1. The first cleavage occurred at 146 minutes after fertilization.

Experiment 3. The eggs collected as in the two previous experiments were kept at 14.2° C and the suspension concentration was 3.33 ml of eggs per 100 ml of suspension. A drop of eggs was removed from the beaker and suspended in 50 ml of fresh sea water. On inseminating the eggs, typical fertilization membranes were formed and 95 per cent underwent normal first cleavage. The first 100 ml sample for chemical analysis was withdrawn 50 minutes after removal of the eggs from the ovary and 91 minutes before fertilization (Table IV). When the eggs in the beaker were inseminated, all formed fertilization membranes. However, these were irregular and elevation from the surface of the eggs was subnormal. The first cleavage occurred at 152 minutes after fertilization, but only 50 per cent cleaved and these ceased to develop at the third or fourth cleavage.

RESULTS

Six experiments were performed on normal eggs, of which two typical ones are presented (Experiments 1 and 2, Table I). An additional experiment was carried out on a concentrated suspension of eggs in which development was abnormal (Experiment 3, Table IV).

Prior to insemination, in the normal unfertilized eggs, in Experiment 1 (Table I and Fig. 1) the slight decrease of inorganic P (column a, Table I) and increase in the quantity of Δ 1 min. P (column d, Table I) observed are within experimental error, while in Experiment 2 (Table I and Fig. 2) a small increase in inorganic P and a decrease in the quantity of Δ 1 min. P occurred. The total quantity of inorganic P after 1 minute's hydrolysis in 0.1 N trichloroacetic acid remained constant in the unfertilized eggs. Following fertilization, however, the inorganic P content decreased markedly, and the quantity of Δ 1 min. P increased. In Experiments 1 and 2, within the

TABLE I[1]

Distribution of P before and after fertilization in the trichloroacetic acid extracts of normal eggs. Quantities in µg P per ml of eggs ± the standard deviation.

	a	b	c	d	e	f
	Inorganic P			Δ1 min. P (column b minus a)	Δ7 mins.P -Δ1 min.P (column c minus b)	Total P
	Before hydrolysis	After 1 min. hydrolysis in 0.1 N TCA	After 7 mins. hydrolysis in 1 N HCl			
Experiment 1						
Unfertilized						
98′ before fertilization	57.6±.6	111±1.1	—	53.4±1 3	—	—
8′ before fertilization	55.1±.6	110±1.1	—	54.9±1.3	—	469±7
Fertilized						
7′ after fertilization	41.1±.4	106±1.1	—	64.9±1.2	—	483±7
82′ after fertilization	25.9±.3	102±1.0	—	76.1±1.1	—	479±7
Experiment 2						
Unfertilized						
31′ before fertilization	83.4±.8	155±1.6	348±5	71.6±1.8	—	—
16′ before fertilization	87.6±.9	156±1.6	344±5	68.4±1.8	188±5	543±8
Fertilized						
6′ after fertilization	56.4±.6	149±1.5	322±5	92.6±1.7	173±5	537±8
39′ after fertilization	56.9±.6	147±1.5	339±5	90.6±1.7	192±5	543±8
70′ after fertilization	36.0±.4	134±1.3	343±5	98.0±1.4	209±5	526±8
107′ after fertilization	41.6±.4	137±1.4	319±5	95.4±1.5	182±5	541±8
150′ after fertilization	33.6±.4	128±1.3	317±5	94.4±1.4	189±5	539±8

TABLE II

Changes in the P fractions, from the moment of fertilization to the time of the last sample, Experiments 1 and 2. Quantities in µg P per ml of eggs.

	a	b	c
	Decrease Inorganic P	Decrease inorganic P after hydrolysis in 0.1 N TCA	Increase in quantity of P liberated after 1 minute's hydrolysis (Δ1 min. P)
Expt. 1	29.2	8	21.2
Expt. 2	58.6	28.5	29.9

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[1] The values in columns a and b are the means of duplicate determinations, while the values in columns c and f are single determinations.

first 6 to 7 minutes after insemination, the inorganic P content fell 25 per cent and 39 per cent respectively (column a, Table I, and solid line with squares, Figs. 1 and 2). Although the greatest changes occurred immediately after fertilization, there were subsequent alterations in the same direction. The total quantity of inorganic P after 1 minute's hydrolysis in 0.1 N trichloroacetic acid showed a moderate decrease after fertilization (column b, Table I and solid line with circles, Figs. 1 and 2), indicating that a portion of the inorganic P had become incorporated in more acid stable compounds. Table II shows the total decrease in inorganic P (from the moment of fertili-

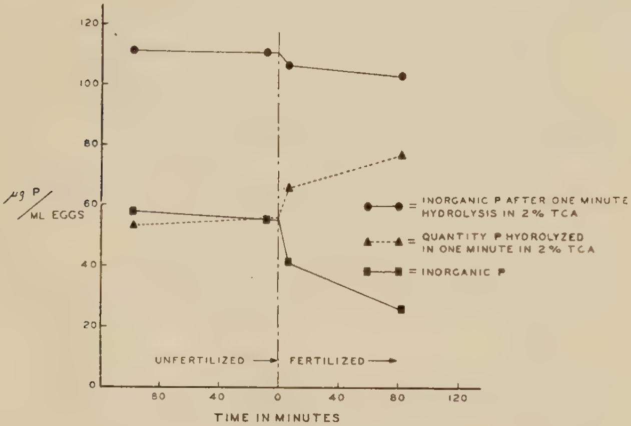

Fig. 1. Experiment 1. Quantities of inorganic P, of P liberated after one minute's hydrolysis and of total inorganic P after hydrolysis for one minute in 2 per cent (0.1 N) trichloroacetic acid at 100° C in the acid soluble extracts of normal *Strongylocentrotus dröbachiensis* eggs. Quantities in μg P per ml of eggs.

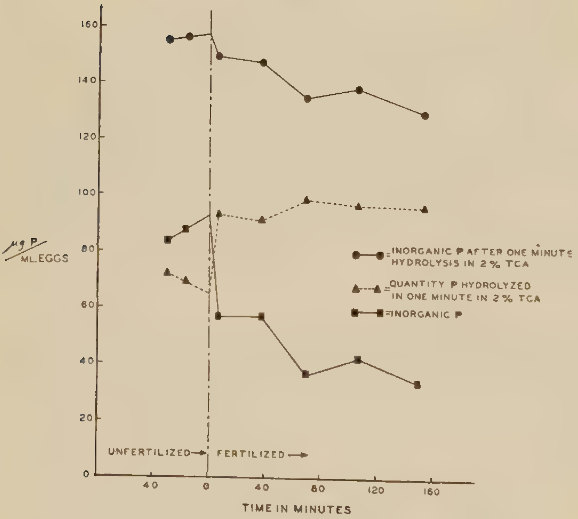

Fig. 2. Experiment 2. Quantities of inorganic P, of P liberated after one minute's hydrolysis and of total inorganic P after hydrolysis for one minute in 2 per cent (0.1 N) trichloroacetic acid at 100° C in the acid soluble extracts of normal *Strongylocentrotus dröbachiensis* eggs. Quantities in μg P per ml of eggs.

TABLE III

Reducing values of the trichloroacetic acid extracts before and after fertilization. Experiment 2.

	Glucose equivalents in µg per ml eggs ± std. dev.	
Unfertilized		
31′ before fertilization	1496 ± 15	
16′ before fertilization	1482 ± 15	
Average		1490 ± 11
Fertilized		
6′ after fertilization	1412 ± 14	
39′ after fertilization	1391 ± 14	
70′ after fertilization	1445 ± 14	
107′ after fertilization	1393 ± 14	
150′ after fertilization	1428 ± 14	
Average		1414 ± 7

TABLE IV[1]

Distribution of P before and after fertilization in the trichloroacetic acid extracts of abnormal eggs. Quantities in µg per ml of eggs ± the standard deviation.

	a	b	d
	Inorganic P		Δ1 min. P (column b minus a)
	Before hydrolysis	After 1 min. hydrolysis in 0.1 N TCA	
Experiment 3			
Unfertilized			
91′ before fertilization	43.8 ± .5	164 ± 1.6	120 ± 1.7
58′ before fertilization	53.1 ± 5	165 ± 1.7	112 ± 1.8
Fertilized			
10′ after fertilization	72.7 ± .7	158 ± 1.6	85 ± 1.8
113′ after fertilization	95.4 ± 1.0	165 ± 1.7	70 ± 2.0

zation to the time of the last sample), the corresponding decrease in total inorganic P after 1 minute's hydrolysis, and the increase in quantity of Δ 1 min. P. It is evident on comparing the values in Table II, columns a and c, that not all the inorganic P which has disappeared can be accounted for as P which has been incorporated in a compound hydrolyzable in 1 minute in 0.1 N trichloroacetic acid at 100° C. The determinations listed in Table I, columns c, e and f were carried out in order to determine where the unaccounted inorganic P had gone. In spite of the considerable variations in these values, due to the high experimental error, the overall constancy of the total P (column f) and the quantity of Δ 7 mins. P - Δ 1 min. P (column e) is evident. These data indicate that the disappeared inorganic P has become incorporated in (a) a compound hydrolyzable in 1 minute in 0.1 N trichloroacetic acid, and (b) in compounds resistant to hydrolysis in 1 N HCl. The data in these experiments are not sufficiently complete to show whether or not cyclic alterations in the quantity of the phosphate fractions occur during the cleavage cycle.

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[1] The values in columns a and b are means of duplicate determinations.

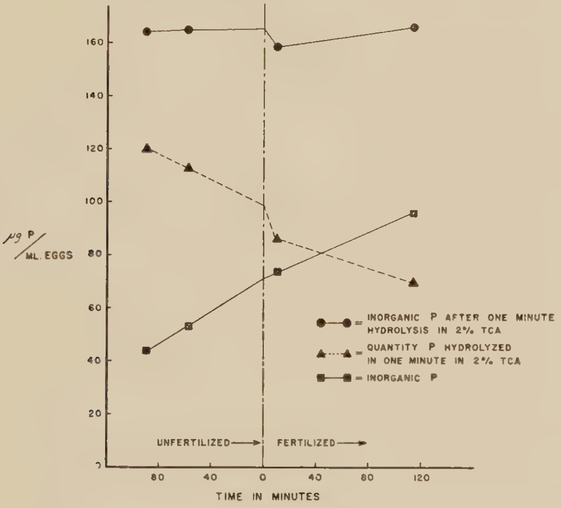

Fig. 3. Experiment 3. Quantities of inorganic P, of P liberated after one minute's hydrolysis and of total inorganic P after hydrolysis for one minute in 2 per cent (0.1 N) trichloroacetic acid at 100° C in the acid soluble extracts of abnormal *Strongylocentrotus dröbachiensis* eggs. Quantities in μg P per ml of eggs.

In Experiment 2 the quantity of reducing substances in the trichloroacetic acid extract was determined using the method of Folin and Malmros (9). The same series of extracts was used as that on which the phosphate analyses had been performed, the determinations being carried out in duplicate. The results, shown in Table III, reveal that fertilization is accompanied by a small but consistent decrease in the reducing values, suggesting a decrease in the quantity of reducing sugars.

Experiment 3 was carried out on a batch of eggs in which the suspension was excessively concentrated and, accordingly, the development was abnormal. The results are shown in Table IV and Fig. 3. The initial quantity of Δ 1 min. P was unusually high. In the unfertilized eggs, a prominent increase of the inorganic P (column a, Table IV and solid line with squares, Fig. 3) and a decrease in quantity of Δ 1 min. P (column d, Table IV, and interrupted line, Fig. 3) was observed. After fertilization the decrease in quantity of Δ 1 min. P, and the increase of inorganic P, continued. The total quantity of inorganic P after 1 minute's hydrolysis in 0.1 N trichloroacetic acid did not decrease following fertilization (column b, Table IV, and solid line with circles, Fig. 3). The inorganic P increased in direct ratio to the decrease in quantity of Δ 1 min. P. These results are in striking contrast to the two previous experiments on normal eggs.

DISCUSSION

The Δ 1 min. P (footnote 1, p. 509) in the acid soluble extracts of unfertilized *Strongylocentrotus dröbachiensis* eggs has been shown to arise from the splitting of arginine phosphate (19). The data indicate that the Δ 1 min. P found in extracts of fertilized eggs also arises from the hydrolysis of arginine phosphate. Creatine phosphate has not been detected either in unfertilized sea urchin eggs (19) or in the fertilized eggs at later stages of development (20). Furthermore, the increase in quantity of Δ 1 min. P could not be due to the formation of glucose-1-phosphate[1] or any other P compound only partially hydrolyzed by an exposure of 1 minute to 0.1 N trichloroacetic acid at 100° C, since the Δ 7 mins. P - Δ 1 min. P (footnotes 1 and 2, p. 509) values remain constant following fertilization (column e, Table I; also see (5)). Under the conditions of the experiment any acetyl phosphate which might have been initially present in the eggs would have been hydrolyzed prior to completion of the phosphate analyses (16).

The experiments, therefore, indicate that a synthesis of arginine phosphate characteristically occurs within the first 6 or 7 minutes after fertilization in normal sea urchin eggs, which have been removed from the ovaries for more than 2 hours.

The increase in quantity of arginine phosphate after fertilization indicates either that free arginine already present in the unfertilized eggs is phosphorylated, or arginine is made available after fertilization. In this connection it is of interest that Örström (21) found an increase in the quantity of free diamino acids after fertilization in *Paracentrotus lividus* eggs.

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[1] Fifty-five per cent of the P of glucose-1-phosphate is liberated after 1 minute's hydrolysis in 0.1 N trichloroacetic acid (19). Hydrolysis for 7 minutes in 1 N HCl causes liberation of all the P combined as glucose-1-phosphate.

No change was noted in the quantity of P in the trichloroacetic acid soluble extracts of the eggs before and after fertilization, through second cleavage, in confirmation of earlier work carried out by Runnström (23) and Zielinski (26). In none of these experiments was phosphate added to the suspensions of developing eggs, the concentration of which ranged from 1 to 10 volumes of eggs per 100 ml of sea water. Since sea water contains between 0 to 60 micrograms P per litre, even if the eggs removed all the phosphate initially present in the suspension fluid, the amount absorbed would be too small relative to the large quantity of P already present within the eggs to be detected. As shown by Brooks and Chambers (2) and Chambers and White (6), fertilized sea urchin eggs accumulate P from the external medium, and studies with P^{32} reveal that over 95 per cent of the entering phosphate is contained in the acid soluble extracts of the eggs. These data indicate that if the concentration of P in the egg suspension was maintained in excess of 20 micrograms of P per litre, an increase in the quantity of P in the acid soluble extract of the eggs would be expected.

The data presented in this paper reveal that following fertilization no change occurs in the quantity of Δ 7 mins. P - Δ 1 min. P in the unfractionated acid soluble extracts. The Δ 7 mins. P - Δ 1 min. P is derived primarily[1], although not entirely, from the splitting of the labile P of adenosine triphosphate. As discussed earlier (5), no measurable alteration occurs in the quantity of adenosine triphosphate in echinoderm eggs following fertilization.

Runnström (23), Zielinski (26) and Örström and Lindberg (22) measured the inorganic phosphate content of the unfractionated acid soluble extracts of *Paracentrotus lividus* eggs before and after fertilization and concluded that no alterations could be detected. Differences in the methods used from those described in the present paper would not appear to account for the differing results. However, it is important to point out that the conclusion reached by the above investigators does not necessarily conflict with our demonstration of a striking decrease in the inorganic phosphate content of *S. dröbachiensis* eggs, in confirmation of the results obtained by Chambers and White (6) on the eggs of *S. purpuratus*. The acid soluble extract of *Paracentrotus lividus* eggs contains a large quantity of inorganic phosphate amounting to approximately 1.25 mg P per ml of eggs (26, 22). This represents an inorganic phosphate content 15 to 30 times that found in the unfertilized eggs of *S. dröbachiensis* and *S. purpuratus* (5, 6).

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[1] After barium fractionation, the Δ 7 mins. P - Δ 1 min. P of the unfractionated extract is recovered primarily in the barium-insoluble fraction as adenosine triphosphate (5), with a small quantity of a compound resembling glucose-1-phosphate and unaccounted Δ 7 mins. P-Δ 1 min. P being recovered in the barium soluble, alcohol insoluble fraction (19). No Δ 1 min. P or Δ 7 mins. P was found in the barium soluble ethanol soluble fraction (19).

If the inorganic phosphate content of *Paracentrotus lividus* eggs decreased after fertilization by the same order of magnitude as observed in the eggs of *S. dröbachiensis* and *S. purpuratus,* not more than a 2 to 4 per cent change could be expected. Such a small alteration is close to the experimental error of the methods used. We carried out inorganic P determinations on the acid soluble extracts of the eggs of *Arbacia punctulata* (4). The inorganic P content of these eggs, 1.12 mg per ml of eggs, is only slightly less than in the eggs of *Paracentrotus lividus.* In two preliminary experiments, following fertilization a slight (2 to 3 per cent) decrease in the inorganic P content was observed. This change, however, was too close to experimental error to be considered valid without further experimentation.

The rapid decrease of the intracellular inorganic phosphate within the first 6 to 7 minutes after fertilization is striking. MacFarlane (17, 18) measured the inorganic P content of the unfractionated trichloroacetic acid soluble extracts of yeast cells. She showed that a decrease in the inorganic P content is detectable within 30 seconds after adding glucose to a yeast suspension previously devoid of an external substrate. Within five minutes after adding the glucose, the inorganic phosphate content of the cell reaches a minimum level amounting to 60 per cent of the initial value. The decrease was due to the incorporation of inorganic phosphate in organic compounds.

The synthesis of arginine phosphate coincides with the early period of augmented respiration observed by Laser and Rothschild (12) in the eggs of *Paracentrotus lividus.* The most probable source of energy for the synthesis of arginine phosphate would be the degradation of carbohydrates. As noted in this paper, a 5 per cent decrease in the quantity of reducing substances was observed in the acid soluble extracts of eggs suggesting a decrease in the quantity of reducing sugars. This result confirms the work of Örström and Lindberg (22) on the eggs of *Paracentrotus lividus,* who observed a 12 per cent decrease in reducing substances. These authors (22, 13, 14) also observed a prominent decrease in the quantity of alkali stable, alcohol precipitable carbohydrate, which they describe as glycogen, within the first ten minutes after fertilization. The results of these investigators should, however, be accepted with reservation, since they used highly concentrated suspensions of eggs (5 to 10 volumes per cent) in which development was admittedly poor (13, 14). An illustration of the anomalies which may result from working with concentrated suspensions in which development was impeded has been presented in this paper. The alkali stable, alcohol precipitable carbohydrate of *Arbacia* (sea urchin) eggs has been identified as glycogen (10). However, Runnström (24) has cautioned that the compound dealt with as glycogen by Örström and Lindberg (21, 13, 14) may actually have represented a mixture with other polymeric carbyhodrates.

Unfertilized *Strongylocentrotus* eggs, exposed to sea water for a period of two hours or longer, present the picture of cells dependent for their survival on a limited quantity of available substrate, such as is true for yeast cells suspended in plain water. The oxygen consumption of the unfertilized eggs is low (1), the inorganic P concentration high, the arginine phosphate content low, and the phosphate content of the eggs remains constant (6) or even decreases (7). Within minutes after insemination, the picture is that of cells with an abundant available endogenous substrate, analogous to that of yeast cells supplied with glucose. The oxygen consumption rises, the intracellular inorganic P decreases, arginine phosphate and acid stable phosphate esters are synthesized and phosphate is accumulated by the cells (2, 6).

SUMMARY

1. In the normal eggs of *Strongylocentrotus dröbachiensis* a rapid increase of arginine phosphate and a decrease of inorganic phosphate have been demonstrated following fertilization. Further alterations in the same direction with minor variations were observed up to the time of first cleavage.

2. The incorporation of inorganic phosphate in arginine phosphate and acid stable phosphorus containing compounds of the trichloroacetic acid soluble extract entirely accounts for the decrease in inorganic phosphate following fertilization.

3. In an experiment performed on a concentrated suspension of eggs, in which development was abnormal, the reverse changes were observed.

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THE STRUCTURE OF CHLOROPLASTS

II. THE FIRST DIFFERENTIATION OF THE CHLOROPLAST STRUCTURE IN *VALLOTA* AND *TARAXACUM* STUDIED BY MEANS OF ELECTRON MICROSCOPY

H. LEYON

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

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The structure of chloroplasts has been dealt with in a number of papers starting from about the middle of the 19th century up to now (11, 17, 15, 14, 18, 5). Yet there is a paucity of material in the literature concerning the formation of the inner structure of chloroplasts. This is to be expected, for if such an investigation is to pretend on completeness submicroscopical structures have to be considered as well. That is the main reason why an electron microscopical investigation of this subject seems pertinent at the present time.

The problem how the chloroplast structures are formed and differentiated during the life of the cell presents several aspects. It is a biophysical and biochemical problem of cytology. Besides, chloroplasts from plants of different phyla differ, and the first stages of the differentiation of the phanerogam chloroplast during cell life, as described in this report, are similar to some chromoplast structures of green algae (14, 18, 6). This fact may contribute to a solution of the phylogenetic problem of the origin and evolution of the chloroplast structures. Furthermore, the chloroplasts have been described as self-reproducing units within the cytoplasm. This aspect has a bearing on the problem regarding the role of non-nuclear cell components in genetics and to some degree also on the question whether or not virus formation occurs in plastids (4).

Nägeli first described how chloroplasts of some algae, ferns and phanerogams multiply through binary fission. Following him several authors observed the same phenomenon in different plants. Schimper (13) gives a review of the literature up to 1885. Chloroplasts shaped like dumbbells had been reported as being in a state of division. It was later pointed out, however, that such forms did not always complete their division and thus did not separate into two parts: the mere occurrence of dumbbell-formed plastids must not be taken as a proof of the reproduction of chloroplasts through binary fission. Reinhard, however, followed the complete division of chloroplasts of several living cryptogams and phanerogams under the microscope and thus verified conclusions drawn from much earlier observations (12). Since the days of Schimper and Meyer up to the present many authors have wrongly interpreted this binary fission as the only way of forming chloroplasts. Long ago, however, Pensa (10) and Lewitsky (2, 3) described how small bodies in the cytoplasm, mitochondria, develop into chloroplasts. Pensa's material was *Lilium, Tulipa* and *Rosa,* that of Lewitsky *Asparagus* and *Elodea.*

With the exception of some occasional observations on the occurrence of "grana" there is only one report on the formation of the inner structure of chloroplasts. It was given by Strugger (16). His results were obtained by light microscopy mainly on fresh material from young leaves of some monocotyledonous plants after vital staining with rhodamin B. Proplastids were observed which were larger than chondriosomes and sometimes contained accumulated starch. In each proplastid Strugger found a disk-shaped inclusion body absorbing the rhodamin. The proplastids multiplied through binary fission, often several at the same time. The rhodamin-stainable disk of each proplastid was stated to participate in the fission of the plastid. The disk was duplicated giving two parallel lamellae, each of which was then found to belong to one of the daughter proplastids. A rapid sequence of reduplication of the small disks is then reported to give the stacks of lamellae typical of the full-sized chloroplasts. The results reported in this paper do not verify Strugger's description.

MATERIALS AND METHODS

Young and rapidly growing leaves were chosen for a study of the formation of chloroplasts. A potted specimen of *Vallota speciosa* (*Amaryllidacae*) which was in a period of intense growth and sprouting happened to be available and was first tried. After its proplastids were found to contain much assimilate an object was sought which might be expected to have little or no accumulation of assimilate in the plastids. *Taraxacum* roots are known to sprout easily and produce leaves even in complete darkness. Since such dark-grown leaves only assimilate substances provided by the root and no carbon dioxide from the air, their plastids might be expected to contain little or no assimilate. Therefore *Taraxacum* leaves grown in the dark were also investigated. The young shoots were some millimeters in length. The blade was developed only as minute lateral expansions of the meristematic tissue of the axis.

The young leaves were cut in small pieces in the fixing fluid and fixed in 0.5 per cent osmium tetroxide at pH 6.9–7.1 for 1 hr. Subsequently they were washed in running water for 2 hrs., then treated with 0.5 per cent formaldehyde for 1/2 hr., running tap water for 1 hr. and with 0.3 per cent chromic acid for 1 hr. After washing and dehydration by means of dioxane they were embedded in a metacrylic resin and cut in a modified Spencer microtome (9) with a glass knife (1). The resin was dissolved from the sections in isoamylalcohol followed by isoamylacetate. Goldmanganin 1:1 was used for the shadow-casting. The sections were studied in a Siemens electron microscope which was run at 90 kV. A primary magnification of 8500 was used.

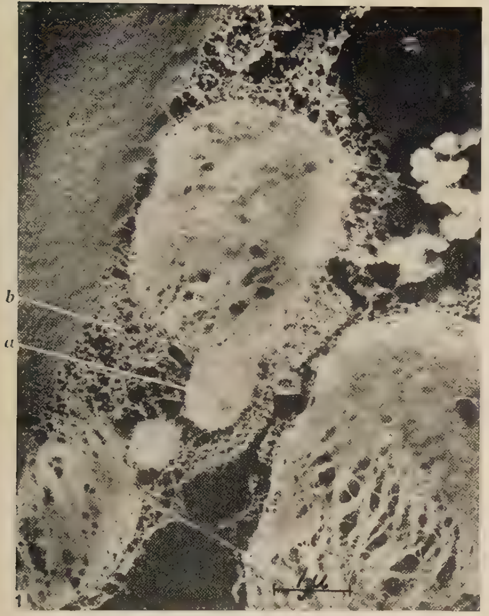
Roots of *Beta sacharifera*, *Nicotiana tabacum* and different species of *Allium* were also studied with respect to assimilate accumulation. The roots had a diameter of 0.5–1.0 mm. Their tips were cut off about 1 mm from their apical ends and the remaining root was cut in pieces of about 1 mm length. These pieces were treated for electron microscopy in the same way as described for the leaves. In the same manner the outer layers of potatoes were studied.

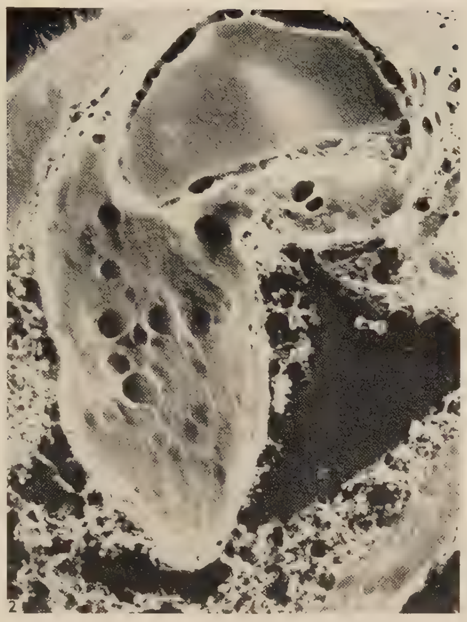
RESULTS

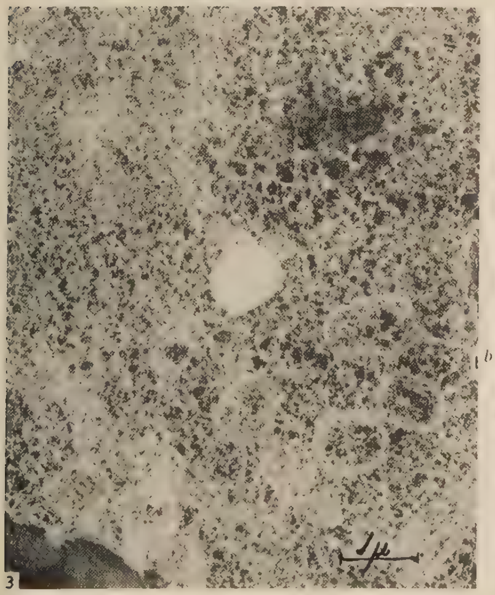
Vallota. The sections of the rapidly growing leaves of *Vallota* showed that the cells of the parenchymatic tissue contained two kinds of bodies. The main difference between them was their "density", i.e. their electron scattering (*a* and *b* in Fig. 1). The dense bodies (*a*) were more varying with regard to their shape than the others. Some of them more or less resembled dumbbells and thus gave the impression of being in a state of division. Dense bodies of varying sizes were found, and of these the large showed the lamellar structure typical of full-grown plastids (Fig. 2). The small dense bodies were thus considered to be proplastids. The smallest of these dense bodies were barely discernible from the structures of precipitated cytoplasm. Of course, the possibility cannot be excluded that only some, but not all, of these dense bodies develop into plastids. The others should then have other functions. Nothing has, however, so far been found indicating this.

The less dense bodies in the sections were more or less ellipsoidal when they were not deformed by accumulated assimilate. This less dense type of structure has been found by the present author also in sections of several roots (*Beta sacharifera, Nicotiana tabacum, Allium sativum*) and in sections of potatoes (Fig. 3). Since the larger ones of this less dense type are in general found to contain accumulated assimilate (compare Fig. 4, b, lower right part), some of the less dense bodies are most likely the loci of enzymes which transform soluble transported material into starch, thus establishing food storage depots. Such enzyme systems are to be expected not only in leaves but also in roots and stems. In food-storing organs they may be expected to occur most frequently.

The sections of the leaves of *Vallota* did not give much information as to the early occurrence of a structure within the plastid. A lamellar inner







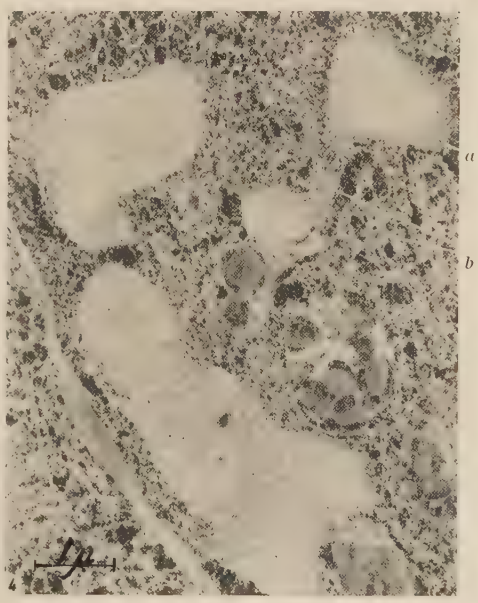
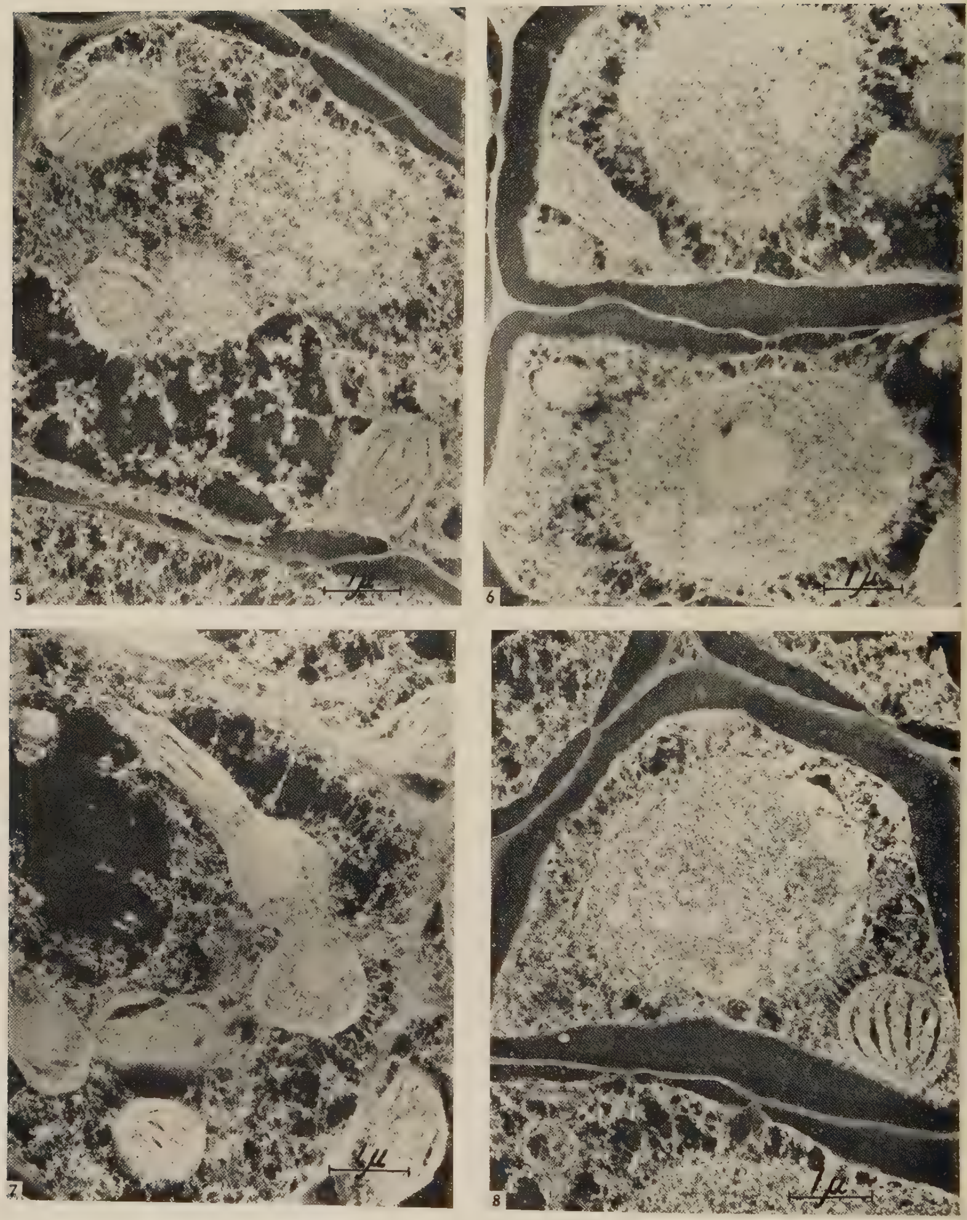


Fig. 1. Section through a young leaf of *Vallota* showing proplastids (a) and less dense bodies (b).

Fig. 2. Section through a young leaf of *Vallota* showing a young lamellated chloroplast with a large accumulation of assimilate.

Fig. 3. Section through the root of *Nicotiana tabacum* showing "less dense bodies" (b) in the cytoplasm.

Fig. 4. Section through a very young shoot of *Taraxacum* with proplastids (a) and less dense bodies (b).


5
1μ
6
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7
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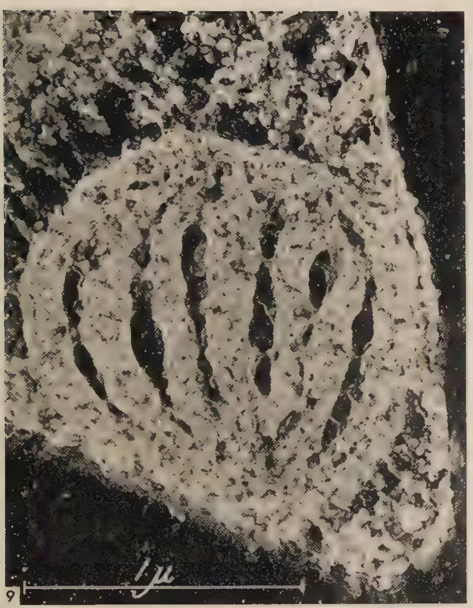
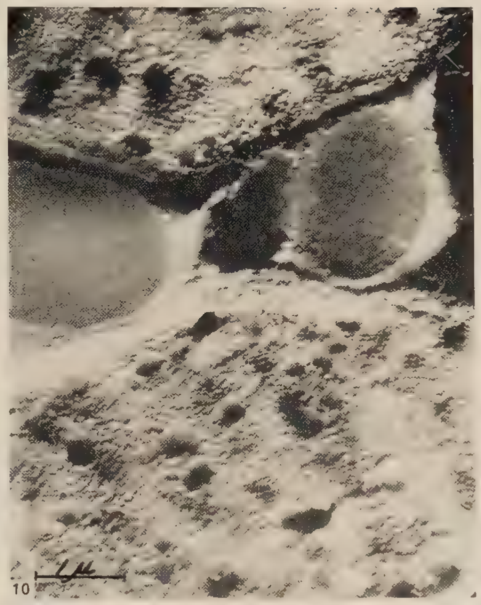
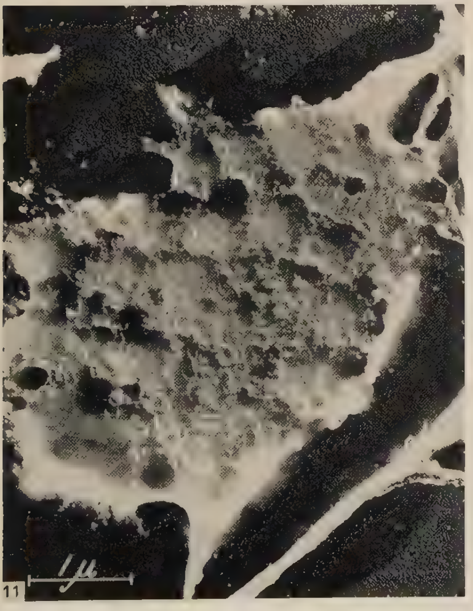
8
1μ

Fig. 5–8. Sections of young leaf of *Taraxacum* grown in the dark, with developing proplastids.







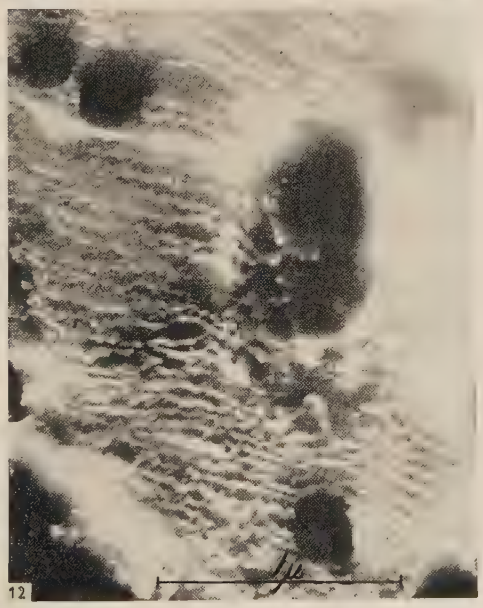


Fig. 9. Detail of Fig. 8 showing a lamellated proplastid.
Fig. 10. Section of a young leaf of *Aspidistra elatior* showing parts of two chloroplasts with accumulated assimilate between. The plastids are lamellated straight through but the laminae are interrupted here and there by accumulations of a substance without a visible structure.
Fig. 11. Section through a chloroplast of a full-grown leaf of *Beta sacharifera*.
Fig. 12. Detail of a preparation like the one of Fig. 10.

structure was found now and then in the dense bodies but always together with accumulated assimilate which obviously more or less concealed the inner structure of the young plastids.

Taraxacum. The leaf primordia of the small dark-grown shoots of *Taraxacum* had their cells filled with the same kinds of bodies in the cytoplasm (Fig. 4) as were found in *Vallota.* The proplastids were, however, free from accumulated assimilate. In the electron micrographs the structure of the young proplastids appears very similar to that of the nuclei. Since the plastids are known to multiply by division, they must be assumed to be the sites of an intense production of protein and thus most likely to contain nucleic acids. This has also been verified by experiments (7, 6). It must therefore be expected that the nuclei material and that of the assimilate-free young plastids are in some degree similar.

In some pictures there seems to be a connection between the proplastids and the nucleus (Fig. 5). However, the data are too few to be taken as an indication of the role of the nucleus in the formation of the plastids.

When the proplastids have reached a certain size they show a laminar structure (Figs. 5 9). The first laminae are few and go straight through the whole chloroplast. One gets the impression that the material of the proplastid is being differentiated subsequently into several parallel laminae.

The first laminae of the young plastid are very thick, about 0.15 μ. In the micrographs each such lamina appears to consist of an outer layer on each side without a visible structure, and between these layers a granulated substance similar to the precipitated cytoplasm outside the plastid.

DISCUSSION

The chloroplasts seem to constitute a most convenient material for a study of the formation of a lamellar system. Beside this, there is the problem of how the number of parallel laminae within the plastid is increased during the life of the plastid. There is further another type of multiplication going on in a direction perpendicular to the one mentioned last, i.e. the multiplication of plastids through binary fission across the laminae. This multiplication seems to be going on during all stages of the development of the plastids.

The findings reported here have a bearing on the much-discussed relation between mitochondria and plastids. For recent reviews the reader is referred to Newcomer (8) and Weier and Stocking (17). Such discussions often suffer from a lack of clearly defined terms. If the word mitochondria is used to include all bodies of a certain size in the cytoplasm, the mitochondria of the leaf cells studied here are obviously of at least two morphological types one of which represents chloroplasts in an early stage of development.

These electron microscopical investigations dealt especially with the first appearance of a lamellation within the plastid. The problem still remains how the stacks of small rounded lamellae typical of the full-sized phanerogam plastid (5) are formed. It is the hope of the present author also to follow that phase of the differentiation of the chloroplast structure more in detail. Even now, however, some conclusions may be drawn regarding the general nature of the whole process of the formation of the chloroplast structure. On account of the restricted character of the present work this outline is to some extent considered hypothetical.

The proplastids which were described above had laminae all the way through. Strugger observed a foliation ("Aufblätterung") through the whole plastids of young leaves when swelling them in alkaline solutions (15) and from this observation assumed that the "grana" were attached to laminae ("Trägerlamellen") extending through the whole plastid. The idea of the grana being suspended on special extended laminae does not seem necessary. Strugger's observation confirms, however, the existence of some such extended laminae. Such laminae have been found by Steinmann *et al.* in *Spirogyra* (14), by Wolken and Palade in *Euglena* and *Poteriochromonas* (18) and by the present author in *Aspidistra elatior* (5), (Fig. 10), and *Clådophora* (6). Since the "extended-laminated" chloroplast is found in the protozoans and algae which have been investigated so far in this respect and in the proplastids of *Taraxacum*, this is very likely a primitive type. The "granular" type of chloroplast may merely be a later stage in the development of the plastid. Since chloroplasts with extended laminae are frequently found to contain accumulated assimilate, the "grana" — i.e. the separated stacks of lamellae — are no necessary condition of assimilation. The formation of stacks of small lamellae may occur when the integrity of the large laminae is interrupted. The micrographs indicate that this is possibly related to the accumulation of assimilate within the plastid (Figs. 10, 11, 12).

The great number of lamellae in some stacks shows that there is an increase in the number of laminae during the life of the plastid. There may also be a production of new lamellae in separate stacks.

This report concerning the formation of plastids is not in agreement with the descriptions and views of Strugger (16). According to him the small disks of the "grana" stacks should be "plastidogens" and as such be carried on from generation to generation through duplications and distribution on daughter plastids. This hypothesis does not seem well founded. In the very young proplastids laminae are formed from a non-differentiated, non-laminated material.

The non-Mendelian heredity of some properties of chloroplasts has no doubt stimulated the opinion that chloroplasts or subunits of chloroplasts are "self-reproducing". These non-Mendelian heredity factors may, however, very well be of a kind that is not detectable by any microscopy.[1]

SUMMARY

1. Sections of young leaves of *Vallota* (*Amaryllidaceae*) grown in the light, and of *Taraxacum* grown in the dark, were investigated with respect to the chloroplast formation.

2. Two kinds of assimilate-containing structures were found; one type is assumed to establish stores of food-stuff, the other to represent chloroplasts in different stages of development.

3. The first ultrastructure to be seen within the proplastid are a few extended laminae.

4. Since the protozoans and algae so far investigated are known to have extended laminae in their chloroplasts, that type of plastid is assumed to be primitive.

5. It is assumed that the "grana", i.e. the stacks of small rounded lamellae, are formed as a later stage in the development of the plastid and may be in relation to the accumulation of assimilate within the plastid. The extended laminae should then be interrupted and a transformation into small rounded parts should occur.

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[1] While this paper was in press a report has been published by E. Heitz and R. Maly in *Ztschr. Naturforsch.*, **8b**, 243 (1953). These authors have repeated Strugger's investigations with the light microscope but were not able to verify his findings concerning the formation of chloroplasts. - It may be noted that Heitz and Maly state that chloroplasts are known to arise only from other chloroplasts. As pointed out above this was never proved and is, in all probability, not true.

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CONTINUOUS LIQUID CULTURE OF THE FUNGUS *OPHIOSTOMA MULTIANNULATUM*

B. VON HOFSTEN, ANGELICA VON HOFSTEN and N. FRIES

Institute of Physiological Botany, University of Uppsala, Uppsala, Sweden

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THE ascomycete *Ophiostoma multiannulatum* (Hedgc. and Davids.) has during the last ten years been the object of genetic and physiological studies at this Institute. For these investigations it has been cultivated either on agar, on which it grows with ordinary hyphae, or in liquid media. In a liquid synthetic medium *Ophiostoma* can grow in the form of hyphae or as budding conidia, much like yeast cells. For certain purposes we wanted to keep *Ophiostoma* growing for a considerable time without the usual accompanying alterations of the medium. A continuous culture was thought to be the best solution to the problem, but for this type of culture a completely homogeneous growth is necessary.

When a number of preliminary experiments had proved that a uniform, conidial mode of growth was possible under certain experimental conditions, various technical arrangements were tried in order to keep the conidial population growing continuously. This problem was also solved and a few experiments were made to illustrate the application of this cultural technique to the fungus *Ophiostoma*.

During recent years several publications dealing with the continuous culture of microorganisms have appeared. The chemostat described by Novick and Szilard (8), and the turbidostatic selector used by Bryson and Szybalski (1) are evidently well suited for genetic studies with bacteria. We have attempted to cultivate *Ophiostoma* in a similar way, *viz.* in glasstubes with air bubbling through, but the growth was not uniform and hyphae tended to adhere to the walls of the tube.

The technique described by Monod (7) was then tried, and has now been used for some months with very good results. The apparatus, which is shown in Fig. 1, has been modified in some respects compared to the original type in Monod's paper. Thus a specially designed peristaltic pump has been used to transport the medium at a constant rate from the storage bottle to the culture flask. The pump is driven by a synchronous motor, the speed of which can be altered with suitable gearings. An inclined tube through the cork at the entrance of the culture flask has also been introduced. Through this one can inoculate the culture and also take samples with the aid of pipettes.

Preliminary experiments showed that the growth was more homogeneous at 30° C than at 25° C, which hitherto has been the standard temperature. The apparatus has therefore been run in a thermostated room with a temperature of 30° ±0.2° C.

Most experiments have been performed with the following medium, which has been developed by Fries (3). Ammonium tartrate 5 g, asparagine 1 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, NaCl 0.1 g, $CaCl_2$ 0.1 g, $ZnSO_4 \cdot 7H_2O$ 4.43 mg, $MnSO_4 \cdot 4H_2O$ 4.05 mg, $FeCl_2 \cdot 6H_2O$ 4 mg, vitamin B_1 100 μg, vitamin B_6 100 μg and distilled water 1000 ml. The carbon source (tartrate and asparagine not utilized), usually glucose, has been autoclaved and added separately.

We are now trying to simplify and vary the composition of the medium, as it is possible that the fungus can dispense with some of the ingredients under the special conditions of continuous culture.

Earlier experiments (3) had indicated that the inclusion of inositol in the medium greatly favoured conidial growth, but it has later been shown that this effect is not very specific. At 30° C and with good aeration a uniform conidial growth is usually obtained both with and without inositol, but the rate of growth is less in the latter case, so we have added 10 mg per litre nutrient in most experiments. The metabolic effect of inositol is still obscure, and a closer investigation of its role as a growth factor for *Ophiostoma* is desirable.

A continuous culture is initiated by transferring a piece of agar-mycelium to a flask or tube with nutrient solution. After two days as shake cultures at 30° C, the growth is often somewhat filamentous. The hypae are then filtered off through cotton, and the conidia centrifuged down and washed with water. The apparatus is autoclaved and mounted and the conidia are pipetted into the culture flask (Fig. 1 on the left side). (The above mentioned procedures are carried out under sterile conditions). Nutrient solution is then introduced from the storage bottle on the right side of the figure. During the entire experiment a stream of moist, sterile air is blown into the culture flask, whereby good aeration is brought about and infection is prevented.

When a given volume of nutrient solution has been added to the flask, the pump is stopped and the culture is allowed to grow for one day. The

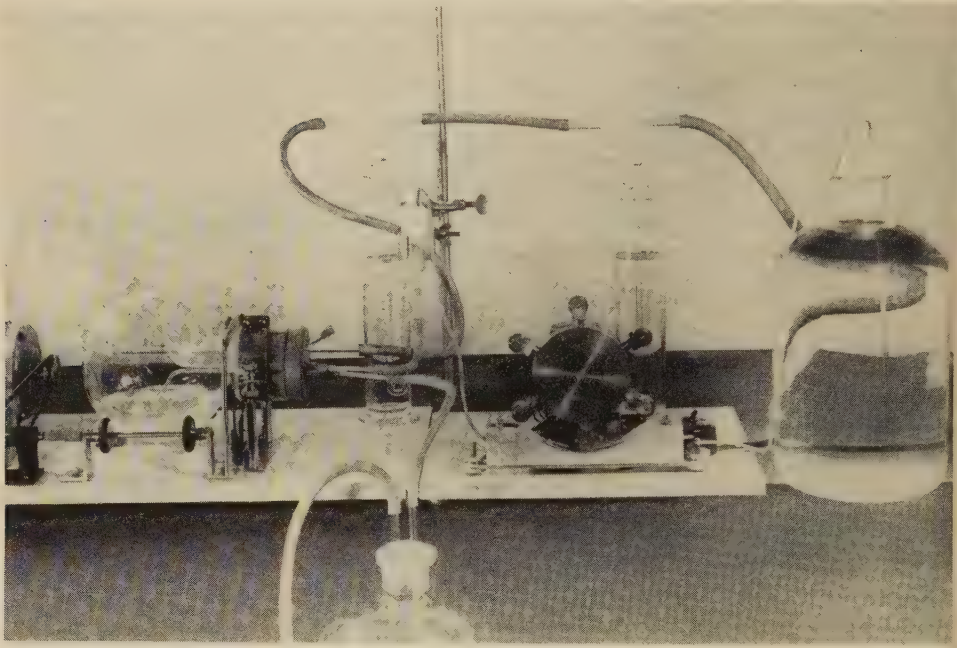

Fig. 1. The apparatus for continuous culture.

pump is then started again and the volume of the culture is now controlled by a tube which leads to the recipient flask (only partly shown on the figure). This is connected to a water suction pump, which is regulated so that it just can draw liquid from the culture without affecting the small positive air pressure produced by the air stream. The culture flask is rotated by a motor at a speed of approximately 150 rpm., and the culture can be maintained for several weeks with the necessary substitution of nutrient.

The growth rate of the cultivated microorganism can be varied with the rate of addition of the nutrient medium, if one of its ingredients, such as glucose, is present in a growth-limiting concentration. This variation can be achieved either by altering the volume of the culture or by changing the speed of the pump. If not changed purposely, the flow rate produced by the pump is constant, and the rubber tubing is very little affected after a week's use.

If an investigation of the chemical composition of the cells produced is desirable, the recipient flask, where the cells are collected, may be kept at a sufficiently low temperature by simple arrangements such as a bucket

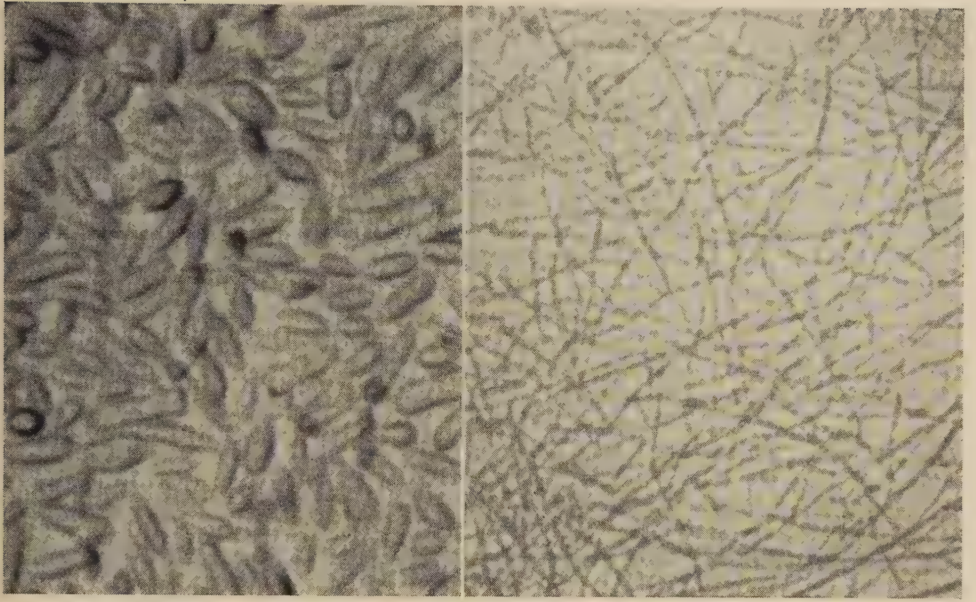

Fig. 2. *Ophiostoma multiannulatum:* Different types of growth. Left: Budding conidia × 2000. Right: Hyphal growth × 700.

of ice. Thus many grams of cells grown at constant rates have been obtained in this way.

For a theoretical treatment of the continuous culture and some of its applications we refer to the papers of Monod (7) and Novick and Szilard (9). If the concentration of one single growth factor is appropriately low the production of cell substances is generally proportional to this concentration. *Ophiostoma* is an unusually economical organism, since on a synthetic medium it may yield as much as 0.6 mg dry cells for every mg glucose added. The individual conidia vary somewhat in size, the average length being about 7 μ, and of these cells there may be as many as $5 \cdot 10^8$/ml. Fig. 2 gives an impression of the difference between conidial and hyphal growth of *Ophiostoma*.

One of our experiments may illustrate how it is possible to keep the fungus growing under varied conditions. *Ophiostoma* conidia are capable of duplication about once every second hour, but in this experiment we had chosen to grow them at a speed of 0.2 generations per hour by fixing a suitable flow rate of the nutrient. When the culture was started, the nutrient contained 0.5 per cent glucose, and at equilibrium we obtained 2.15 ± 0.03 mg dry weight per ml corresponding to $2.3 \cdot 10^8 \pm 0.4 \cdot 10^8$ cells per ml. After three days we changed over to a new nutrient with 0.25 per cent glucose. The course of the experiment can be followed in Fig. 3.

Although attacked by many investigators (4, 5, 6, 12), the problem of maintaining a continuous culture of a microorganism under controlled conditions and with a constant cell density, has not been solved satisfactorily until recently. Monod (7) and Novick and Szilard (8), who published their results almost simultaneously, are the first to have worked out the theory of the method. The continuous culture of bacteria is many times of great value in biochemical and genetic work, but it has certain limitations. In some respects *Ophiostoma* offers obvious advantages over bacteria, since the cells are easier to manipulate and the yield is much greater. The lack of sexual reproduction in most bacteria is an obvious drawback. With *Ophiostoma* it is possible to analyze the material genetically in a more satisfying way since it is a heterothallic, sexual organism, like the related *Neurospora*.

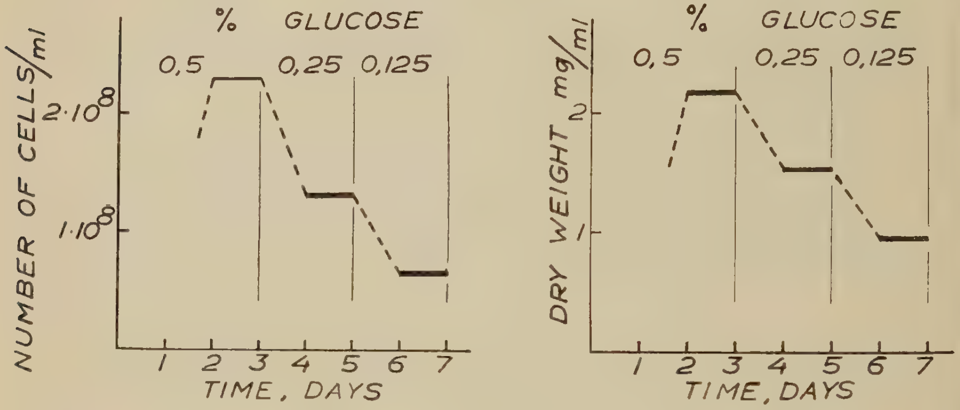

Fig. 3. Continuous culture of *Ophiostoma multiannulatum* with different amounts of glucose. The unbroken lines indicate stable cultures.

Many interesting problems in microbial genetics can evidently be solved elegantly by continuous culture, as has been shown by Novick and Szilard (9, 10, 11) and Bryson and Szybalski (1). The technique also promises to give valuable information on the physiology and biochemistry of cell division. Our laboratory is also attacking selection, adaptation and evolution in different strains of *Ophiostoma*, and for these problems the described apparatus is an invaluable tool.

SUMMARY

A method is described by which it is possible to maintain a fungus under conditions of constant and continuous growth. By altering the rate at which fresh nutrient is added to the culture, the growth rate of the organism can be controlled. Since, hitherto, similar methods have been used exclusively in bacterial studies, the advantages of using a fungus as the experimental material are discussed.

The present investigation has been supported by grants from "Harald och Greta Jeanssons Stittelse", Stockholm, and Eli Lilly and Co., Indianapolis, Ind. We are indebted to Mr. B. Pettersson for valuable assistance in constructing the apparatus for continuous culture.

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BRIEF NOTES

THE THICKNESS OF THE SEA URCHIN FERTILISATION MEMBRANE

J. M. MITCHISON[1]

Department of Zoology, University of Cambridge, England

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This note describes measurements with the electron microscope of the dry thickness of the sea urchin egg fertilisation membrane, and also a method of estimating the wet thickness by flotation measurements of the density.

The dry thickness of the fertilisation membrane was given by Harvey and Anderson (1) as 250 Å from absorption comparisons on electron microscope photographs. Such comparisons, however, are only approximate and it was therefore decided to measure the thickness by the more accurate method of shadowing.

Fertilisation membranes were stripped off the eggs of *Psammechinus miliaris* by forcing them through narrow mesh bolting silk, and were then fixed in 4 per cent formalin. The fixed membranes were washed in distilled water and dried on EM grids. The dried membranes were then shadowed at 15° with gold-palladium and photographed with a Siemens and with an R.C.A. electron microscope by Mr R. H. Horne at the Cavendish Laboratory, Cambridge. One of the photographs is shown in Fig. 1. and it can be seen that the membrane shows no visible structure.

The negatives were enlarged, and the shadow width was measured at the edges of what appeared to be single thicknesses of the dried membrane. Owing to the rather uneven background and the thinness of the membranes, it was difficult to get a very accurate figure for this shadow width. The approximate width under both microscopes from several membranes is given below:

	Shadow width in mm.	Magnification of enlargements.	Membrane thickness in Å.
Siemens	1–2	35 700	75–150
R.C.A.	1	23 000	115.

In some of these photographs there appeared to be a slight thickening of the membrane at the edge, so these figures may err on the large side. It seems reasonable to take a figure of 100 Å for the approximate thickness of the dried fertilisation membrane. This agrees fairly well with recent measurements made with an interference microscope (4).

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[1] Now at Department of Zoology, University of Edinburgh, Scotland.

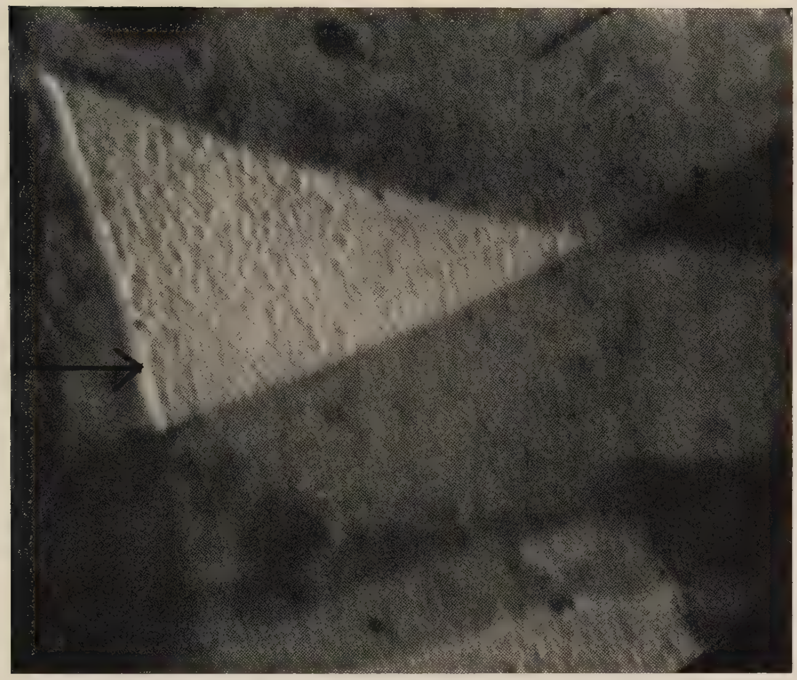

Fig. 1.

If the degree of hydration of the membrane could be found, it would be possible to estimate the wet thickness of the membrane from the figure for the dry thickness given above. This could be done by flotation measurements of the density (i.e. seeing whether wet membranes rose or fell in liquids of different density), provided liquids could be found which did not alter the density of the wet membranes. Liquids immiscible with water were useless, as the membranes retained a film of water round them on immersion. So, also, were salt solutions, as they penetrated the membranes very quickly, and gave the density of the membranes as that of the protein only, instead of the protein plus water. This pointed to protein solutions, and a penetration test was therefore carried out with a concentrated egg albumen solution (about 30 per cent). The birefringence of a torn membrane was measured immediately after placing in the albumen solution, and then at intervals of a minute for ten minutes. There was no change, indicating that neither penetration of the albumen, which would have diminished the form birefringence (owing to the high refractive index of the solution), nor dehydration, which would have increased it, had taken place to a significant extent. This solution, therefore, was suitable for flotation.

A suspension was made of the torn membranes of *Psammechinus miliaris* by grinding fertilised eggs fixed in formalin between two slides. A drop of this suspension was added to albumen solutions of varying concentrations, and centrifuged for two minutes. Samples of the liquid at the top and bottom of the centrifuge tubes were then examined under the microscope, to see whether the membranes had risen or fallen. Eventually, two albumen solutions of slightly different concentrations were found, in one of which, solution A, the membranes rose, and in the other of which, solution B, they fell. These two solutions were then tested in the same way with small cubes of gelatin of known concentration. It was found that in solution A, 27 per cent gelatin (by weight) fell and 26 per cent rose, and that in solution B, 24 per cent fell and 23 per cent rose. Since it is reasonable to assume both that the membrane is mainly composed of protein, and that this protein has approximately the same density as gelatin, therefore the membrane must be between 26.5 and 23.5 per cent protein by weight — with an average of 25 per cent. Assuming the protein has a density of 1 · 27 (that of gelatin), the membrane will therefore contain roughly 20 per cent protein by volume.

If this figure of 20 per cent protein by volume is combined with a dry thickness of 100 Å, we arrive at a figure of 500 Å for the thickness of the wet membrane. This is certainly low, but it is not unreasonable. The thickness must be below the limit of resolution of the microscope (c. 2500 Å), since the inner and outer edges are not resolvable even under the most favourable conditions. At the same time the membrane should be quite visible under the microscope. The minimum detectable thickness of an opaque line is 180 Å or less (2), and a large spherical membrane should be much easier to see, both because light is reflected off its surface, and because it curves round above and below the focal plane.

An attempt was made to apply to the fertilisation membrane the detailed polarised light analysis which was used on the red cell ghost (3), but it was eventually found that the resolving power of the optical system was not high enough to distinguish between a very thin membrane of high birefringence and a thicker membrane of low birefringence. It should be mentioned that Runnström, Monné and Wicklund (5) from polarised light measurements, give a figure of 1 μ for the thickness of the fertilisation membrane. However, the calculations by which this figure is derived are erroneous, and a detailed criticism has been given in Swann and Mitchison (6).

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THE NUCLEOLAR SIZE IN THE LIVER CELL OF RATS FED HIGH AND NON PROTEIN DIETS

U. STENRAM

Department of Histology, University of Lund, Sweden

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Caspersson and co-workers (1) claim that when protein synthesis in the cell is increased, the nucleolus becomes larger. There are a few exceptions in that some embryonic cells have large nucleoli even when their protein synthesis is not increased. These cells are, however, prepared for a period of rapid growth and are thus not regarded as true exceptions (1).

Two workers submitted the liver cell of rat to investigation from this point of view. Stowell (5) fed rats a low protein diet for three or five weeks and found the nucleoli in the liver cells to be as large as those of rats fed a high protein diet. When the experimental period was extended to 54 or 92 days, the nucleoli of the protein-depleted animals became larger than those of the protein-fed animals. Stowell claimed that there was a decrease in protein synthesis in the livers of the low protein-fed animals, a conclusion drawn from the decrease in plasma protein that followed protein restriction. According to Stowell, Caspersson's above-mentioned assertion should therefore not always hold for the liver cell. On the other hand, the work of Lagerstedt (3) confirmed Caspersson's statement. Lagerstedt fed rats high and low protein diets for maximum 18 days and found the nucleoli of the high protein fed animals to be larger than those of animals fed low protein diet. He found a decrease in liver protein of low protein fed animals and consequently assumed that there was a decrease in protein synthesis in the liver cells.

The discrepancy between Lagerstedt's and Stowell's results on nucleolar size may be due to differences in technique, such as differing diets, experimental times and methods of measurement. Due to the great importance of the possible significance of nucleolar size *the author will reinvestigate the influence of high and non protein diets on liver nucleolar size.*

Material and methods. Six male and six female white rats (*Mus norw. alb.*) were weaned at 21 days of age, at an individual weight of about 36 g. They were equally divided into three experimental groups and given different amounts of protein in their diets. Group 1 was given 22 per cent crude casein; group 2, 3 per cent; and group 3, 0 per cent.

The diets consisted of 2 g peanut oil, 1 g cod liver oil, 4.5 g salt mixture (2), casein (see above), sucrose ad 100 g, 0.02 g α-tocopherol acetate, and 0.17 g of the mixture 1 mg thiamine, 2 mg riboflavin, 1 mg pyridoxin, 10 mg calcium pantothenate, 10 mg nicotinamide, 5 mg inositol, 30 mg p-aminobensoic acid, 100 mg choline chloride, 0.2 mg pteroylglutamic acid, 0.05 mg biotin and 14.2 mg 2-methyl-1.4-naphtoquinone. Each animal got a daily subc. injection of 0.3 γ vitamin B_{12}. All animals were fed *ad libitum*, given tap water and sacrificed after ten days. A fourth group consisted of two males and two females sacrificed at 21 days of age.

A piece of the middle lobe of the liver was fixed in Susa and embedded in paraffin. Methyl benzoate was used as intermedium. Sections were cut 15 μ thick and stained with gallocyanin-chromalum. The diameters of 75 nucleoli from each liver were measured with a 15 × compensation ocular micrometer and an oil immersion objective 90 × N.A. 1.25. Some nucleoli were not round and were not considered. The mean nucleolar volume was determined for each experimental group, each group thus totalling 300 observations. The number of nucleoli per nucleus was determined by counting the nucleoli in 100 nuclei from every animal. Only such nuclei were considered whose entire volume was found within the section. Some sections were stained after previous treatment with ribonuclease.

Result and discussion. The results are given in Table I. There is a significant difference between group 1 and either of the groups 2 and 3 ($P < 0.001$). Between groups 2 and 3 ($0.2 < P < 0.3$) there is no difference. The difference in size as well as in variation of size between group 1 and groups 2 and 3 is clearly visible. There is no difference between the groups with regard to the number of nucleoli per nucleus. This calculation was made to avoid the objection that a decrease in nucleolar size of the high protein fed animals might be due to an increase in number of nucleoli per nucleus. The results of group 4 eliminate the objection that the non protein fed animals might remain in a stage with larger nucleoli.

TABLE I

Group	Nucleolar size in μ^3	Number of nucleoli per nucleus
1	2.4 ± 0.07; $s = 1.2$. $n = 300$.	2.23 ± 0.08; $s = 0.17$. $n = 4\ (\times 100)$.
2	3.1 ± 0.09; $s = 1.6$. $n = 300$.	2.15 ± 0.03; $s = 0.05$. $n = 4\ (\times 100)$.
3	3.3 ± 0.10; $s = 1.7$. $n = 300$.	2.21 ± 0.07; $s = 0.13$. $n = 4\ (\times 100)$.
4	1.7 ± 0.04; $s = 0.7$. $n = 300$.	1.91 ± 0.06; $s = 0.12$. $n = 4\ (\times 100)$.

There was, subjectively evaluated, no difference in the intensity of the basophilia of the nucleoli of the various experimental groups. Minor differences in nucleic acid contents are, however, difficult to establish with basic dyes. Therefore no conclusions should be drawn from this observation. It was extremely difficult to ascertain any differences in the amounts of nucleolo-associated chromatin between the various experimental groups.

There were very few or no basophilic inclusions in the liver cell cytoplasm of the low and non protein fed rats, but in the high protein fed animals the inclusions were abundant. This fact indicates an increased protein synthesis in the livers of the latter animals in relation to the former. The problem may, however, not be so simple. An animal on a restricted protein diet will nevertheless try to maintain its protein metabolism. The stores in the liver will rapidly be consumed, explaining the disappearance of the basophilic inclusions, but there still remain other protein resources in the body. Proteins less necessary for the maintenance of life might be transformed in the liver into more essential proteins. Such processes might strain the protein-synthesizing apparatus of the liver more than the normal metabolism of proteins from the food, and the nucleolus should then become larger. There would be no time for storing proteins or nucleic acids in the liver cell cytoplasm. If this hypothesis is true it would be possible to demonstrate an increase in the nucleic acid contents of these nucleoli by means of ultraviolet absorption measurement. Stowell, however, did not find an increased amount of nucleic acids in the nucleoli of protein-depleted animals. The tasks of the liver though are numerous and there may be some process which would cause an increase in the nucleolar volume without increasing the amount of nucleic acids. This assumption is supported by very small nucleoli being present in starved animals (3, and own observations). Such rats seem to be as protein-depleted as animals fed protein-free diets. In the latter case, however, the diet may be such that it stimulates nucleic acid metabolism in the liver (4).

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ROLLER TUBE TISSUE CULTURE APPARATUS

W. C. LISTER and C. F. DORÉ

National Institute for Medical Research, Mill Hill, London, Great Britain

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Recent requirements for Roller Tube tissue culture equipment by Units of the Medical Research Council have been met by a design utilising readily interchangeable drums. The machine finally evolved will accept pairs of drums varying widely in axial length so that different lengths of culture tube may be used. Drum diameters must be approximately the same but different diameters of tubes and bottles may be accommodated by different arrangements of holes. Variation in the angle of tilt of the vessels is obtained by adjusting screws which tilt the whole machine.

Dimensions are such that the whole unit with two drums will fit into an incubator of internal dimensions 20″ × 20″ × 24″ high, and either drum may be removed complete with its tubes without disturbing the other or stopping the machine. A drum, when removed from the machine, may be laid on the bench like an ordinary test tube rack for loading or cleaning. More important still, the media may be replenished in any tube without removing it from the drum, which, after recharging, may be slipped back into the machine again without stopping it. The holes which accept the tubes are pierced in a regular pattern of lines each lettered at its ends. The position of any tube is thus readily identifiable and, since the drive is such that balance is unimportant, the tubes may be assembled in logical groups and spaces may be left at will.

The construction of the machine and the manner in which it is used should be clear from the illustrations supplemented by the following notes.

The drums are friction driven and are retained in position in the machine solely by their own weight. Each drum sits on a pair of wheels and a pair of rollers which are carried by two parallel spindles. Wheels and rollers are rubber tyred and the wheels which act as drivers are keyed to the spindles. The rollers are free. The spindles are chain driven from one motor, a Drayton Regulator Co. Type RQR speed 1 rev. in 1.75 mins. With 1/1 chain sprockets, a driving wheel diameter of $3\frac{5}{8}$ ins. and a drum diameter of 15 ins. this gives a drum speed of 1 rev. in $7\frac{1}{4}$ mins.

The motor and the bearing brackets for the spindles are carried on an angle framework which sits on a pair of fixed feet and a pair of screw feet for tilting. Some tilt is necessary to ensure that the drums retain their position against the flanges of the driving wheels and that the tubes retain their positions in the drums, and also to keep the culture medium away from the stoppers of the tubes.

Two horizontal rails are fitted which protect the mechanism from objects pushed up against it on the bench, serve as handles when moved, and also provide a rest for the drums when putting them into or taking them out of the machine.

The motor, driving sprocket and chain (Renold 8 mm) are located under a hinged sheet metal cover at one end of the machine. This cover carries the switch, fuse, Fig. 1 (right). Complete instrument with two drums in position.

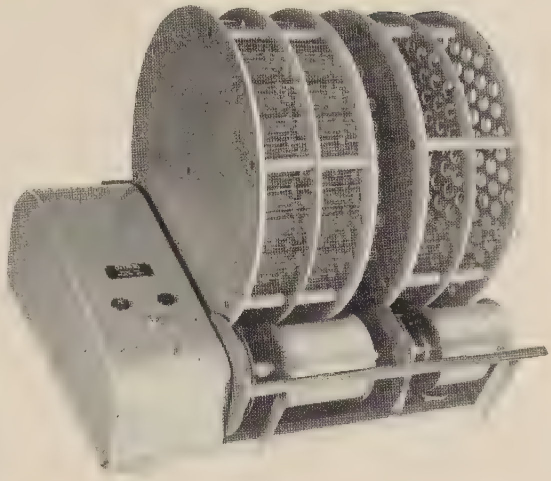
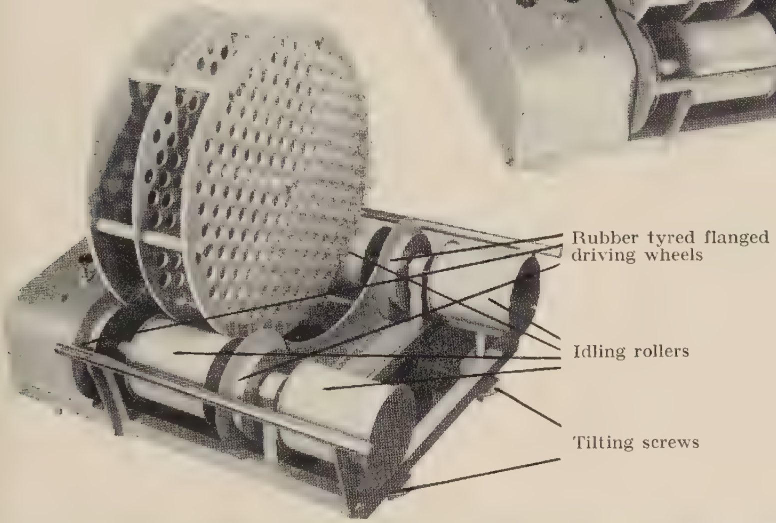


Fig. 2 (below). One drum removed to show constructional details.


Rubber tyred flanged driving wheels
Idling rollers
Tilting screws

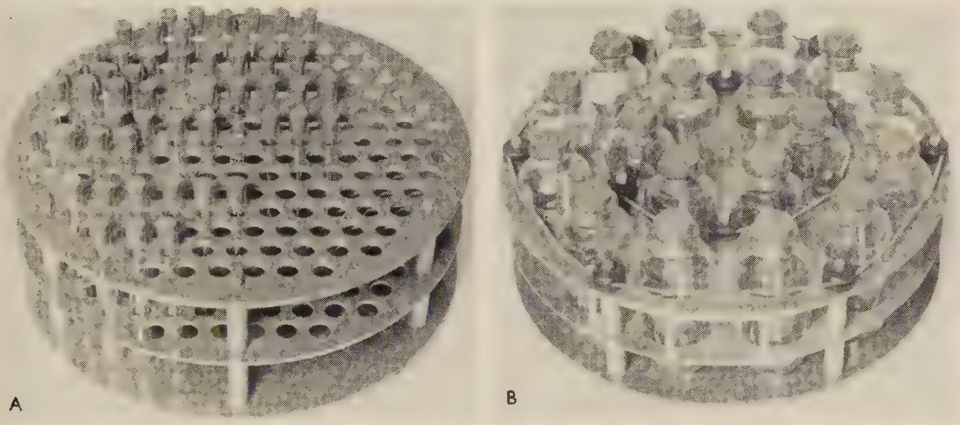


Fig. 3. Two types of drum which have been used. (A) takes 162 $\frac{5}{8}''$ dia test tubes; (B) takes eighteen 250 ml centrifuge bottles.

pilot light and supply plug so that all the more vulnerable parts are protected but may easily be reached by undoing one screw and lifting the cover. One chain runs over all three sprockets and tensioning adjustment is obtained by slotted fixing holes for the motor. The actual drums consist of three discs of fabric bakelite, two of them pierced with holes appropriate to the vessels to be carried. The discs are separated by duralumin pillars. Paper base material would probably be better than fabric bakelite and in an earlier machine Perspex was used. This however is not so strong for handling and the essence of the machine is that the drums should be handled.

Drums have been made to accommodate various sizes of tubes and also 250 ml centrifuge bottles. The readiness with which one type of drum may be removed and another substituted is an important feature in this connection, and the long idling rollers allow considerable variations in axial length of the drums.

In the first machine that was made, too many tubes were packed into one drum and difficulty was experienced in getting tubes in and out. With the drum diameter adopted of 15 ins., the number of $\frac{5}{8}$ in. diameter tubes was reduced from 210 to 162 and the equipment was then much more reasonable to handle.

One drum is shown in Fig. 3. It will be noticed that springs are provided to hold the bottles in position. These are "spring curtain rod" stretched so as to apply some pressure to the sides of the bottles and thus provide additional restraint.

Thanks are due to the Director of the National Institute for Medical Research for permission to publish details of an instrument which was designed and produced in the Instrument Division of that Institute.

Acknowledgment must also be made of the help of Dr. Goffe of the Central Public Health Laboratory, Colindale, for whom the first of these instruments was made.

MICRO-ALIGNMENT OF ALPHA RADIOAUTOGRAPHS BY MEANS OF A CONTACT REPLICA OF THE TISSUE PATTERN PRINTED WITH VISIBLE LIGHT

H. J. SCHAEFER and A. GOLDEN

U. S. Naval School of Aviation Medicine, Pensacola, Florida, and Department of Pathology, Emory University School of Medicine, Emory University, Georgia, U.S.A.

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Radioautography of histological sections is a method of paramount importance in locating radioactive elements deposited within tissues. To ensure accurate alignment, different procedures of permanently mounting specimen and photo-emulsion have been designed. However, the technique of permanent mounts implies, besides some inconveniences in processing, the basic disadvantage that the density of the silver grains in the emulsion sometimes impairs seriously the microscopic examination of the specimen. To avoid this obstruction for alpha radioautographs, we have developed a method which permits not only separate microscopic examination of the stained section and of the autograph but full maintenance of an accurate micro-alignment.

Open spaces are present in most histological sections. Some are of natural origin, e.g., the lumina of capillary vessels, whereas some are artefacts due to microtome cutting or to removal of soluble cell material in processing. Our method takes advantage of this pattern of holes in the tissue section for alignment by printing a contact replica of this pattern on the nuclear emulsion with visible light.

For the radiographic exposure, the stained sections are brought in direct contact with a nuclear emulsion in the usual manner. To avoid drying and shrinking of the sections we found it advantageous to keep the sandwiches under colorless mineral oil (heavy oleum petrolatum) during the radiographic exposure. After the latter is finished the still intact sandwich is exposed briefly to visible light. Hematoxylin-eosin stains give a light replica of satisfactory contrast without filter. However, the contrast can be greatly increased by using the Kodak Wratten B-filter, green, No. 58. Feulgen stains are more difficult to handle. It is advisable to make the stain as deep as possible, but even then a filter is required. We obtained best results with Kodak Wratten A-filter, red, No. 25, using Kodak NTB-emulsion.

The photomicrographs in Figs. 1 and 2 show two tissue sections and their radioautographs selected from our study on the distribution of thorotrast in the animal body. Fig. 1 shows a section of bone marrow. The oval-shaped holes in the tissue, appearing as heavily blackened areas in the light replica, represent fat cells, the fat having been dissolved by xylene in processing. The remaining intact tissue consists of normal bone marrow cells and, to the right, of a group of macrophages filled with thorotrast. The alpha activity is seen in the radioautograph and can be exactly

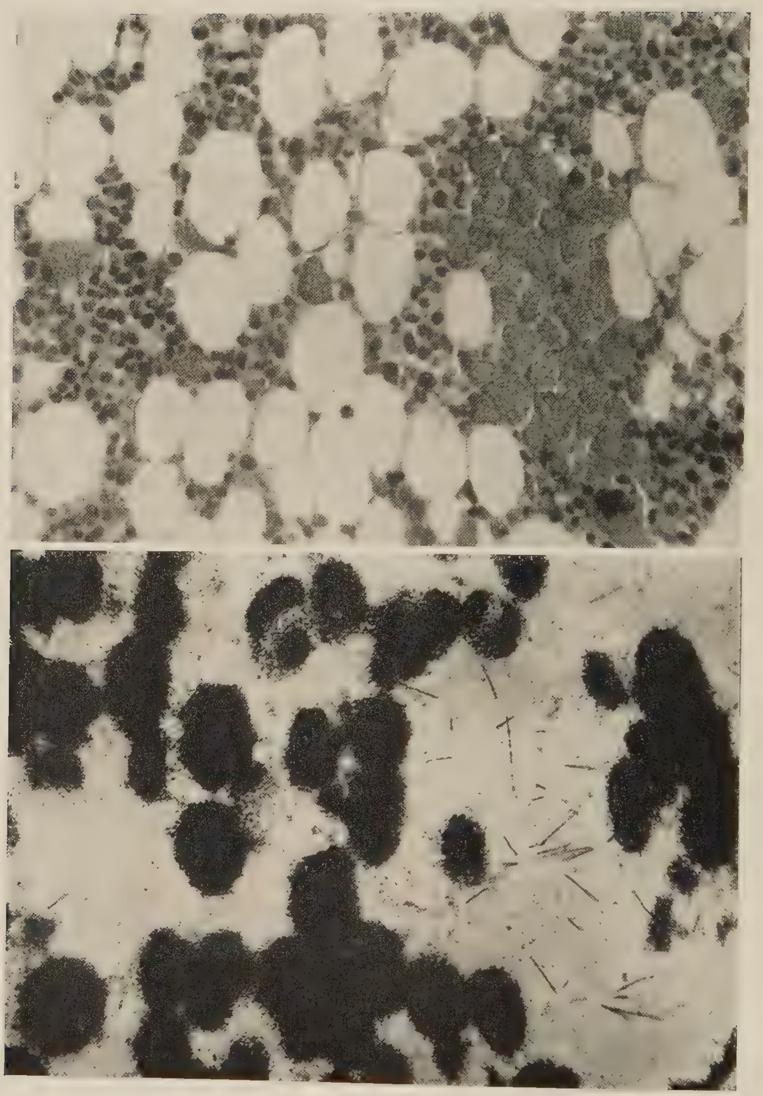

Fig. 1. Top: Section of bone marrow of thorotrast-injected rabbit. Alcohol fixation, Hematoxylin-Phloxine, 6 μ. Visual field 312 × 228 micra.

Bottom: Simultaneous light contact replica and radioautograph of section. Large dark oval areas produced by transmitted light. Tracks produced by thorium alpha particles emitted from thorotrast inclusion.

Transparent light areas brought about by removal of fat during preparation. Marrow cell nuclei visible throughout the section. Stippled grey area (top, right of center) is intracellular thorotrast, corresponding to region of alpha tracks (bottom).

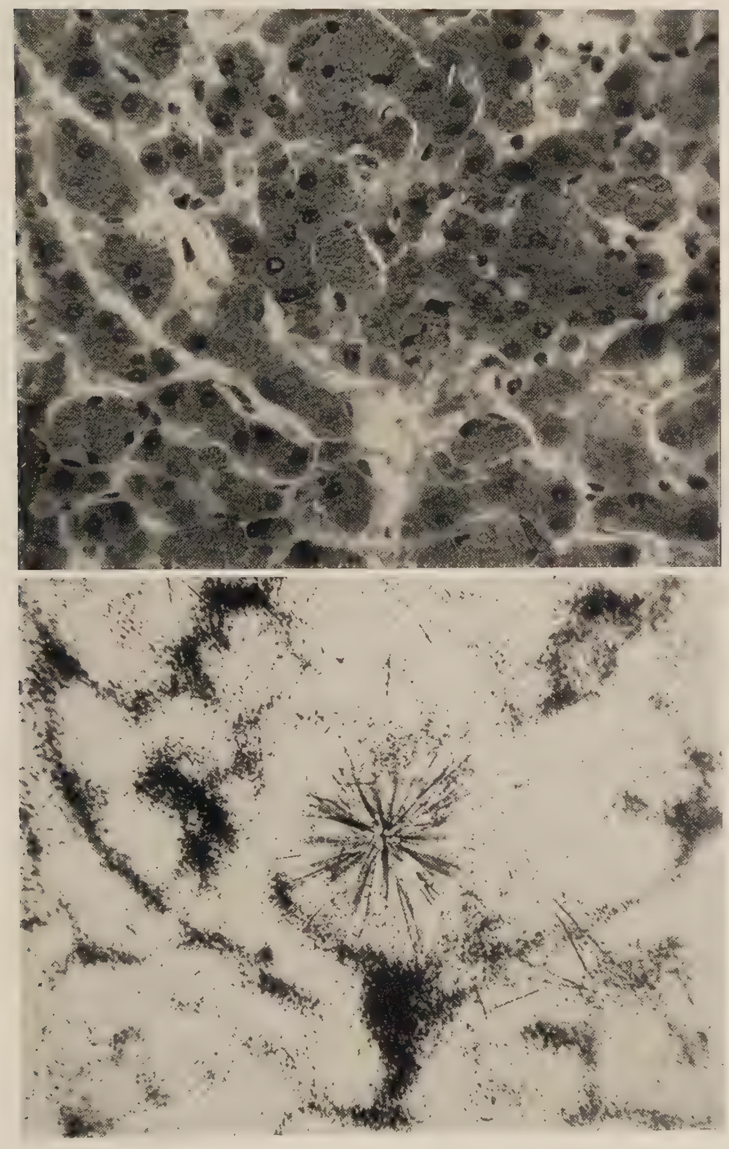

Fig. 2. Top: Section of liver of thorotrast-injected rabbit. Formaldehyde fixation, Hematoxylin-Phloxine, 6 μ. Visual field 305 × 240 micra.

Bottom: Simultaneous light contact replica and radioautograph of section. Dark clusters produced by transmitted light. Tracks produced by thorium alpha particles.

Light replica pattern permits localization of star center in the Kupfer cell directly in center of top photograph, completely filled with thorotrast (compare characteristic texture of thorotrast with Fig. 1).

determined by track count. Fig. 2 shows a section of liver. The tissue section shows many of the previously mentioned cleavages which furnish excellent alignment references in the contact replica on the radioautograph. The submicroscopic source of alpha activity close to the center can be clearly traced to a Kupfer cell which has phagocyted a large amount of thorotrast.

THE CONTROL OF NUCLEAR AND CYTOPLASMIC SYNTHESIS BY THE NUCLEO-CYTOPLASMIC RATIO IN *SACCHAROMYCES*[1]

C. C. LINDEGREN and S. A. HADDAD

Biological Research Laboratory, Southern Illinois University, Carbondale, Ill., U.S.A.

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When a cell from a cake of bakers yeast is introduced into nutrient medium, it shows no perceptible increase in volume but after 80 minutes it begins to bud. Minor fluctuations in the density of the cell occur during the period preceding the appearance of the first bud. Since it has been demonstrated (see below) that all the materials required to produce a new nucleus are synthesized during this period, it is inferred that cytoplasmic synthesis does not occur until after the bud appears.

Experimental. One-half gram of yeast from the center of a fresh cake was suspended in 50 ml of Burkholder's nutrient medium (without asparagine) in a 300 ml Erlenmeyer flask, placed on a mechanical shaker and incubated at 30° C. Under these conditions, Sarachek (to be published) reported that budding appeared to be synchronous throughout the first budding cycle. The densities of individual cells were measured at 15-minute intervals. Five ml aliquots were centrifuged and the residue washed quickly three times with ice-cold water. The residue from the last washing was resuspended and washed three times in approximately 1 per cent KCl solution. This solution was used throughout the density measurements (2). The viscosity of the KCl solution was determined by an Ostwald-Cannon-Fenske viscosimeter over a range of temperature from 25° C to 30° C and the viscosity values plotted in poises. A similar curve of density and temperature using a Leach specific gravity bottle served as a reference for viscosity and density constants of the KCl solution and to calculate the density values of the individual cells.

Results. The average densities of the cells during the budding cycle were determined in a series of experiments one of which is presented in the table. A definite pattern of fluctuation occurs during the period preceding budding. Each figure represents the average of from twenty to thirty observations selected at random; no significant variation in the values was found in any single aliquot.

It is not feasible at present to measure density after the cell begins to bud since laws governing the behavior of the rates of fall have not been determined for objects resembling budded cells.

Conclusions. Data on the kind of synthesis which precedes the appearance of the bud, during the period of constant volume, are available from two sources: 1) Ogur, Minckler and McClary (to be published) analyzed yeast cells for their desoxyribosenucleic acid content during the budding cycle. If each cell with a bud were counted

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TABLE I

Densities of yeast cells at different times during the period of constant volume.

	15 min.	30 min.	45 min.	60 min	75 min.	90 min.
Density	1.050 ± .008	1.054 ± .002	1.082 ± .016	1.049 ± .007	—	—
Per cent of cells with buds	None	None	None	None	40	90

as two cells (even when the bud was so small that it was only barely perceptible), the DNA content per cell was a constant showing that at the first appearance of the bud the DNA has already doubled, and, therefore, that the synthesis of all the DNA necessary for the formation of the new nucleus had occurred. 2) Sarachek (to be published) recorded the effect of ultraviolet irradiation on inactivation during the budding cycle. When the cells were irradiated at various stages during the period of synchronous division, at the appearance of the first bud, the cells contained twice as many targets sensitive to ultraviolet as the original unbudded cells.

Determinations of the density of the cell during the period preceding the appearance of the first bud have revealed fluctuations between 1.05 and 1.08, or somewhat less than 3 per cent of the total weight of the cell. Since the volume remained constant, little, if any, increase in total weight can have occurred. The data cited above indicate, however, that a complete new nuclear apparatus was synthesized during this period.

These data support the following hypothesis concerning the significance of the ratio of nucleus to cytoplasm in the budding cycle: When the resting cell with a single nucleus and a full complement of cytoplasm is placed under conditions favorable for growth, sufficient nuclear material is synthesized to produce a second nucleus and the ratio of nucleus to cytoplasm becomes twice its original value. At this point the synthesis of cytoplasm is initiated and progresses until the ratio again reaches that of the resting cell. During growth of the yeast cell, the bud is in constant communication with the mother cell; the mother cell does not increase in volume because its cell wall is sufficiently rigid to limit expansion to the extension of the wall of the bud. A sufficient amount of material enters the bud to provide all the structures essential for a new cell. When the bud reaches the volume of the mother cell, the "resting cell" ratio of nucleus to cytoplasm is reestablished in the complex. The connection between the two cells is closed—synthesis of nuclear material occurs, and new buds appear on both mother and daughter cell.

Summary. Determination of the densities of individual yeast cells during the period preceding the formation of the first bud revealed that the densities fluctuated only slightly while the volumes of the cells remained constant. Other data indicate that the new nucleus is synthesized during this period. It is inferred that synthesis of cellular material is controlled by the nucleo-cytoplasmic ratio: synthesis of nuclear material occurs before the bud appears and synthesis of cytoplasm occurs while the bud is enlarging.

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GALLOCYANIN-INDIUMALUM

ELECTRON STAINS I

G. F. BAHR

Institute for Cell Research, Karolinska Institutet, Stockholm, Sweden

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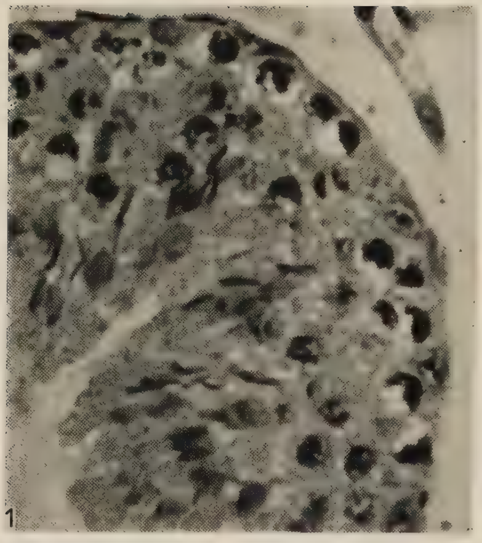
In studying the fine structure and properties of nuclear elements it was desirable to use a stain which would make possible observation in the light microscope and also introduce a heavy metal component to give sufficient contrast in sections for electron microscopy.

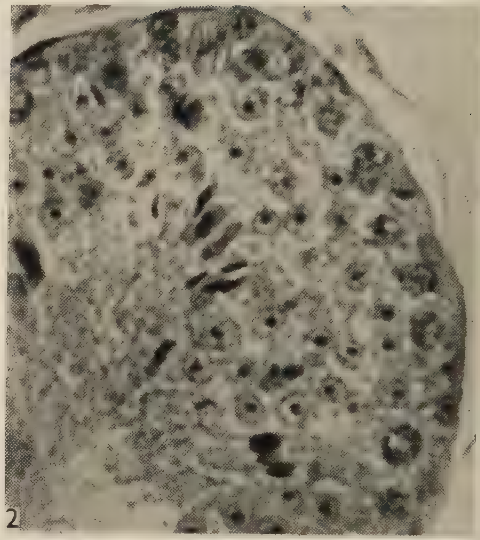
The specificity of the well-known gallocyanin-chromalum stain for nucleic acids has recently been questioned (1–4). Whether this stain may be used for quantitative work or not, there is doubtless a rather good selectivity at a proper pH. This selectivity and the ability of the oxazines to form dye-lakes with certain metals also make them suitable as *electron stains*. In the course of a study of different metals as alum-lake forming components indium was of particular interest because of its high atomic weight (114.76), which gives it electron contrast, and its easy accessibility in pure form. Results with other metals will be reported later in this series. This article is concerned with the property of indium of increasing the colour of the gallocyanin-lake stain.

The dye-solution is prepared in the following way: Mix 3 g $In(SO_4)_3$ pro analysi with 3 drops conc. sulphuric acid. Dilute with 200 ml double distilled water. Add 20 g $(NH_4)_2SO_4$, boil and cool. The solution must be clear. Add 3.5 g gallocyanin, stir well and boil for two hours in a reflux apparatus. Filter the cool solution and save the filter residue. The pH of the dye-solution, as measured with a glass electrode, is 1.6.

The dye-solution is used undiluted and can be kept unchanged for two weeks. After that time a slow hydrolysis with precipitation of dark flakes occurs and the surface of the dye is covered by a thin layer which tends to adhere to slides and samples. It is advisable to filter the solution each time before use. Freshly prepared solutions give best results. Used and old solutions which do not contain other metals than indium and alkalies may be saved and boiled to dryness with conc. sulphuric acid. Then the indiumsulfate will easily be extracted by boiling diluted H_2SO_4. After filtering and washing the In precipitates as hydroxide upon the addition of NH_4OH. $In(OH)_3$ is not soluble in an excess of NH_4OH or NH_4Cl. The In may be estimated gravimetrically as In_2O_3 by heating the hydroxide. In_2O_3 dissolves readily in hot diluted sulphuric acid and can again be used for the dye-lake preparation.

The microphotographs show sections of Carnoy-fixed guinea pig testis tissue, 8 μ





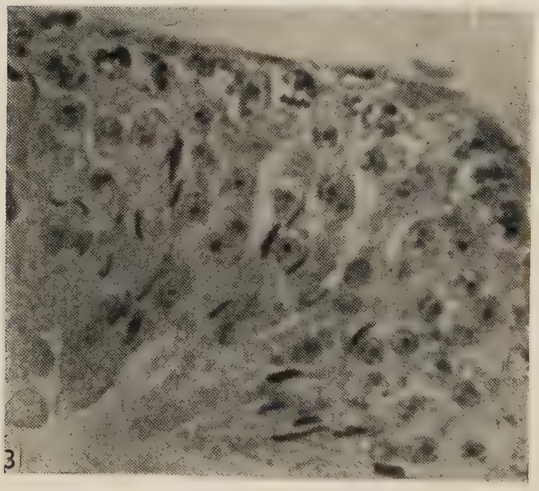


Fig. 1. Gallocyanin-indiumalum stain.
Fig. 2. Gallocyanin-chromalum stain.
Fig. 3. Gallocyanin-aluminumalum stain.

thick, deparaffinized twice in xylol and twice in benzol. The sections were then run through an alcohol series to dist. water and stained in the dye-solutions for 24 hrs. They were washed for 24 hrs. in running tap water, passed through alcohol, benzol and xylol and sealed in Canada balsam. Fig. 1 shows a section stained with gallocyanin-indiumalum, Fig. 2 gallocyanin-chromalum, and Fig. 3 gallocyanin-aluminumalum. The effect of the increasing atomic weight on the staining intensity is obvious. The Co-staining increases too, but is relatively weaker with In. The staining is not effected if the sections are immersed in the following liquids for 48 hrs.: conc. $(NH_4)_2$

SO_4, NaCl, acetic acid, trichloracetic acid, formalin, abs. alcohol, benzol, xylol, butyl alcohol, isoamylacetate and methyl- and butylmetacrylate. Others that have only a slight decolourizing effect are: sodium acetate, urea, dil. mineral acids and glycerin. The use of gallocyanin-indiumalum as an electron stain will be reported elsewhere.

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THE SUPRAVITAL STAINING WITH JANUS GREEN B OF MITOCHONDRIA IN THE RETINAL RODS OF THE GUINEA-PIG EYE

L. G. ELFVIN

Department of Anatomy, Karolinska Institutet, Stockholm, Sweden

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As has been previously shown (4) when analysing the ultrastructure of the retinal rods of the guinea-pig eye, the so-called ellipsoid in the inner segments consists of a dense aggregation of rod-shaped mitochondria. The identification of this structure as mitochondria was based on the similarity of the ultrastructure as compared with the mitochondria of pancreas cells (4) and of tubular cells of the kidney mitochondria (6).

Despite the convincing evidence presented by Sjöstrand it seemed desirable to check the mitochondrial nature of the "ellipsoid" by means of the conventional supravital staining with Janus Green B.

Lewis (2) used Janus Green staining in a study on the role of the mitochondria in the development of the visual cells of chick embryos. Granules which were stained with Janus Green were observed in the inner segments of rods and cones of developing as well as of adult cells.

The basal rod structure of the tubular cells of the mouse kidney is considered to represent the mitochondria of these cells. This assumption has been based mainly on their characteristic form and size and on unspecific staining properties.

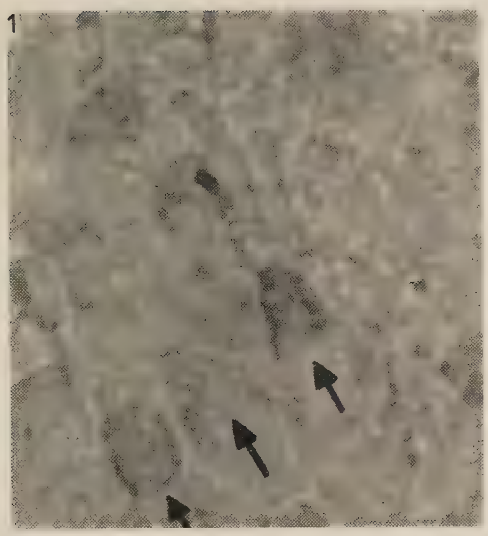
When applying the Janus Green technique supravitally Oliver (3) observed, however, that long rodlets in the tubular cells of rat kidney were stained. He considered them to correspond to the rodlets of these cells which are generally classified as the kidney mitochondria.

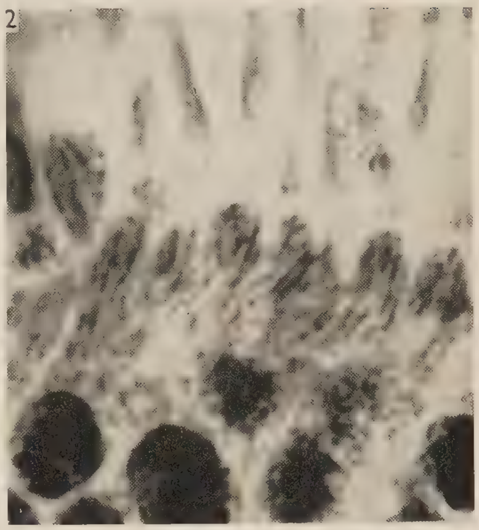
In the present study Janus Green staining of kidney tissue was merely used as a basis for comparison with the conditions in the rods.

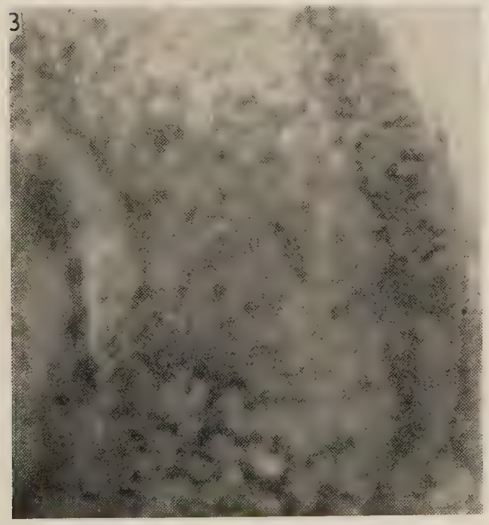
Material and Methods. Albino guinea pigs weighing about 300 g were used for studying the retinal rods. They lived under ordinary daylight conditions and were killed by decapitation. The retina was dissected free as quickly as possible and transferred into a Ringer solution at about +10° C. Fragments of the retina were carefully separated by means of glass needles under a dissecting microscope and immersed in a drop of Janus Green B (diazin green) on a microscope slide. The stain was dissolved in a Ringer solution at a dilution of 1: 18000. When adding 0.88 M saccarose solution (1) the staining effect was not improved.

After 15 min. the specimen was covered by a cover glass. The maximum intensity of the staining is reached after another 10–15 min.

Animals weighing 20–30 g were used for the study of the tubular cells of the mouse







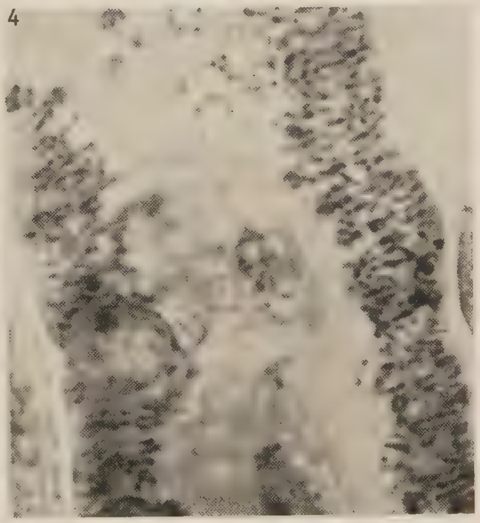


Fig. 1. Teased preparation of guinea-pig retina stained supravitally with Janus Green B. The stained inner segments of the retinal rods are indicated by arrows. Magnif. 2400 ×.
Fig. 2. Microtome section through the guinea-pig retina stained with Heidenhains ironhematoxylin stain. The striated appearance of the rod inner segments corresponds to a staining of the inner segment mitochondria. Magnif. 2400 ×.
Fig. 3. Teased preparation of a tubule from the mouse kidney stained supravitally with Janus Green B. Along the right side the tubular mitochondria are well stained. Magnif. 2400 ×.
Fig. 4. Microtome section through the mouse kidney showing a part of a proximal convoluted tubule with the mitochondria stained with Heidenhains ironhematoxylin. Magnif. 2400 ×.

kidney. The tubules were isolated through gently teasing a thin slice of kidney tissue with glass needles.

For comparison frozen-dried retinas and kidney tissue were stained with Heidenhains ironhematoxylin.

Results. The retinal rods. As soon as the cover slip has been mounted a zone immediately proximal to the outer segments appears blue green. When examining at a higher magnification it is obvious that this zone corresponds to the inner segments, the differential staining of which is maximal within 25–30 min. At that time rod-shaped and sometimes granular structures within the inner segments are well stained.

The color of the stain changes into violet and becomes more diffusely spread over the retina. At the time of maximal staining it appeared especially clear that the stained granules were concentrated to the distal part of the inner segments (Fig.1).

In frozen-dried retinas rod-shaped structures in the inner segments could be stained by means of Heidenhains ironhematoxylin (Fig. 2).

In the tubular cells of the kidney the basal zone appears intensely blue green 10–15 min. after the application of the cover glass. At a high magnification the basal rods appear differentially stained (Fig. 3). Sometimes the individual rods are not distinguished, the basal part of the cells then appearing more uniformly stained.

Fifteen to 20 min. after covering the tissue fragments with the cover glass the color changes to red. At about the same time the rods become indistinct and finally vanish. After about 1 1/2 hour the staining has faded completely.

Using the ironhematoxylin stain on frozen-dried kidney tissue the basal rods appear very distinctly as shown in Fig. 4 which is presented for comparison.

The mitochondrial nature in the inner segments of guinea-pig retinal rods has thus been verified by the differential staining of the inner segments with Janus Green B. This is in agreement with the observations of Lewis (2) on chick visual cells as far as the Janus Green staining is concerned. She did, however, not recognize that the Janus Green stained granules correspond to particles that can be identified as mitochondria in fixed material. The observations on kidney mitochondria confirm the results obtained by Oliver (3).

The author's thanks are due to Prosector F. S. Sjöstrand on whose suggestion this investigation was undertaken.

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SUBMICROSCOPIC ORGANISATION OF SOME LAYERED LIPOPROTEIN STRUCTURES. (NERVE MYELIN, RETINAL RODS, AND CHLOROPLASTS)

J. B. FINEAN[2,3], F. S. SJÖSTRAND[1], and E. STEINMANN[1]

Depts. of Anatomy[1] and Physical Cell Research[2], Karolinska Institutet, Stockholm, Sweden and Dept. of Pharmacology[3], Univ. of Birmingham, England

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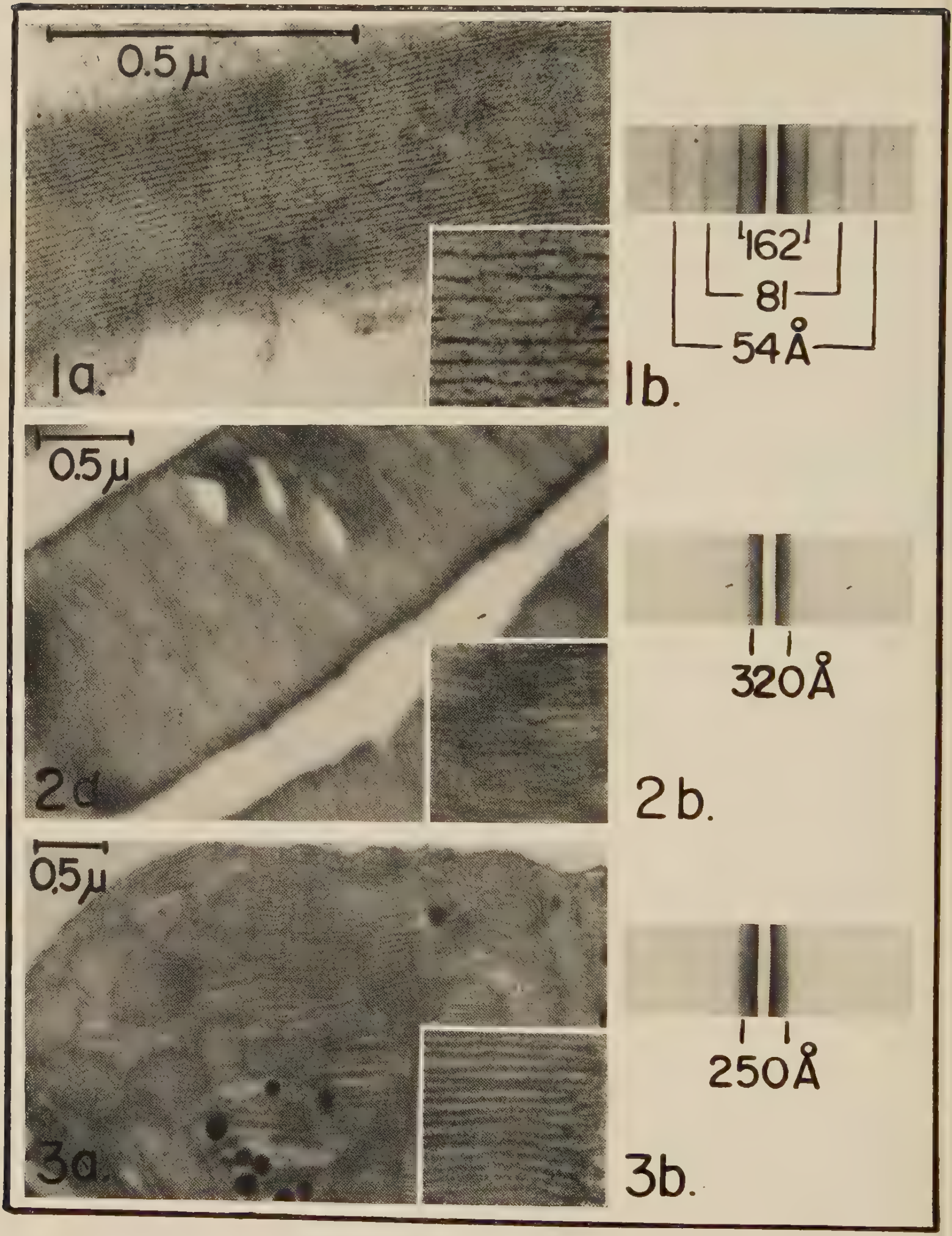
In the electron micrographs of osmium tetroxide-fixed preparations of peripheral nerve myelin (1, 5), retinal rods (4, 6), and chloroplasts (3, 7, 8), the osmium staining has revealed regular layered structures which have many features in common. An X-ray diffraction study (2) on the effects of osmium tetroxide on nerve myelin and related lipid structures has indicated a stabilization of the ultrastructure without any gross changes. The intensity of diffraction is increased by the addition of osmium to the system, and marked changes in the relative intensities of the orders of diffraction indicate depositions at specific levels in the structure so as to emphasise the fundamental repeating unit. The dimension of the latter is reduced by about 15 Å in the wet OsO_4-fixed preparation, and by a further 25 Å or so on drying. The fundamental repeating unit for moist OsO_4-fixed mouse sciatic nerve (Fig. 1 b) is 162 Å. The electron micrographs of nerve myelin (Fig. 1 a) show a concentric layered structure in which the repeating unit of dense and lightly stained layers corresponds approximately to one half of the fundamental X-ray unit, the probability being that there is some asymmetry in the distribution of osmium in consecutive layers which has not yet been resolved by the electron microscope. The sharpness of the X-ray reflections and the rapid decrease in intensity with increasing order of diffraction, are in keeping with a system containing a limited number of regularly spaced units.

So far, diffraction patterns have been obtained from retinal rods and chloroplasts only after OsO_4-fixation. Fixed guinea pig rods gave well-defined low angle reflections indicating a fundamental structural repeating unit of about 370 Å for the wet preparation. The electron micrographs (Fig. 2 a) showed a regular layering throughout the length of the rod, and a repeating unit (6) (Fig. 2 a inset) more complex than that in nerve myelin but giving a spacing corresponding very closely to the fundamental X-ray unit (370 Å). Perch rods showed a very similar structure, but with an E.M. repeating unit somewhat higher than for guinea pig rods. This repeating unit corresponds almost exactly to the X-ray diffraction spacing (Fig. 2 b) of 320 Å obtained from preparations of OsO_4-fixed perch rods.

The low angle reflections obtained from chloroplasts were in general not so well-defined as those from retinal rods and nerve myelin, and a possible explanation of this finding is immediately obvious from the electron micrographs (Fig. 3 a). The layering is regular only within limited areas scattered through the structure, and the number of ordered diffracting units in some preparations would appear to be insuf-


0.5 μ
1a.
162
81
54 Å
1b.
0.5 μ
2a
320 Å
2b.
0.5 μ
3a.
250 Å
3b.

ficient to produce sharp X-ray reflections. A clear diffraction band at 250 Å (Fig. 3 b) has been obtained from OsO_4-fixed chloroplasts (in sucrose) of *Aspidistra*, and this spacing is in exact agreement with the repeating unit of the layers shown in the electron micrographs.

These preliminary observations show that in all three systems the repetition of structural units can be sufficiently regular and extensive to produce precise X-ray reflections. The organisation of the lipid and protein layers can be varied so as to give structural repeating units of different dimensions which can be distinguished crystallographically, and these variations can be seen in the distribution of osmium deposits in the electron micrographs. In the case of the retinal rods and chloroplasts, there is almost exact agreement between the X-ray diffraction spacings and the dimensions of repeating units seen in the electron micrographs. This could be a coincidence, for the specimens used for the two methods might not be expected to be strictly comparable. However, it is possible that the plastic used in the embedding procedure for electron microscopy might replace the water normally in the structure without affecting the dimensions of the structural units appreciably. It is not yet certain with what type of chemical groups the osmium is associated, but it is possible that some information on this point may be obtained from a detailed correlation of the E.M and X-ray diffraction data on the ultrastructure with the variations in chemical composition among and possibly within these systems. The unfixed retinal rods and chloroplasts appear to have a relatively low stability, so that they lose their organisation fairly rapidly after isolation. This makes the recording of the diffraction pattern of the fresh material rather difficult, and even with fixed material the preparation of specimens for diffraction and electron microscopy must be carried out under carefully controlled conditions.

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Fig. 1 a. Electron micrograph of thin section of OsO_4-fixed and embedded mouse sciatic nerve. × 87,000 (Inset × 260,000).

Fig. 1 b. Low angle X-ray diffraction pattern of OsO_4-fixed mouse sciatic nerve.

Fig. 2 a. Electron micrograph of thin section of OsO_4-fixed and embedded guinea pig retinal rods. × 27,000 (Inset × 55,000).

Fig. 2 b. Low angle diffraction pattern of OsO_4-fixed perch retinal rods.

Fig. 3 a. Electron micrograph of thin section of OsO_4-fixed and embedded chloroplasts from *Aspidistra*. × 20,000 (Inset × 100,000).

Fig. 3 b. Low angle diffraction pattern of OsO_4-fixed chloroplasts from *Aspidistra*.

A HIGH RESOLUTION ULTRA-MICROSPECTROPHOTOMETER FOR LARGE-SCALE BIOLOGICAL WORK

T. CASPERSSON, F. JACOBSSON, G. LOMAKKA, G. SVENSSON,
and R. SÄFSTRÖM

Institute for Cell Research, Medical Nobel Institute, Karolinska Institutet, Stockholm, Sweden

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In ultra-microspectrophotometric work for quantitative purposes the measuring instruments must fulfil a large number of requirements. These have been described earlier (1). Hitherto, these procedures have been used mainly for the study of the nucleic acid and protein metabolism, in the spectral range between 2500 Å and 3300 Å. For such problems, it is essential to work with high resolutions, i.e., better than 1 μ. This is because the individual cell structures to be studied are of this order of magnitude. Moreover—owing to the lack of homogeneity of biological material—the use of too large measuring areas causes errors in the measurements. In strongly absorbing material, these may be exceedingly large. On the other hand, several reasons make it unsuitable, in the case of average biological specimens, to work with measuring areas having a diameter of less than 0.3 μ.

A universal ultra-microspectrophotometer for use in the range between 2000 Å and short-wave infrared has been described earlier (2). This instrument permits considerable variations to be made in the measuring technique, in order to suit the individual characteristics of different kinds of biological material. An instrument for scanning measurements and an automatic analyzer for the measured curves have also been reported (4). This group of instruments includes, in addition, an arrangement for determining the light dispersion around the measured object (3).

The universal microspectrophotometer is, however, unnecessarily complex for many of the problems concerned with the cellular nucleic acid and protein metabolism. Furthermore, it is essential in such cases to have an apparatus which is simple to operate, has a high degree of constancy and stability, and permits rapid work. This is because biological specimens are, as a rule, easily damaged by radiation and other procedures. Moreover, all such investigations require large series of measurements. An ideal instrument should—despite its intricate mechanism—be able to be operated by a biological worker who, as a rule, has only limited experience of complex photoelectric machines.

In constructing the instrument described below, all these factors were taken into account. The basic equipment is specially designed for work in the spectral range 2500 Å to 3300 Å and in the visible region. In these regions, the resolution and accuracy are the same as in the large universal spectrograph, both for point measurements and for scanning measurements. The apparatus is, however, far more rapid and simpler to operate. It can easily be adapted for use for the same purposes as the universal spectrophotometer. Thus, it may be utilized for work in other wave-

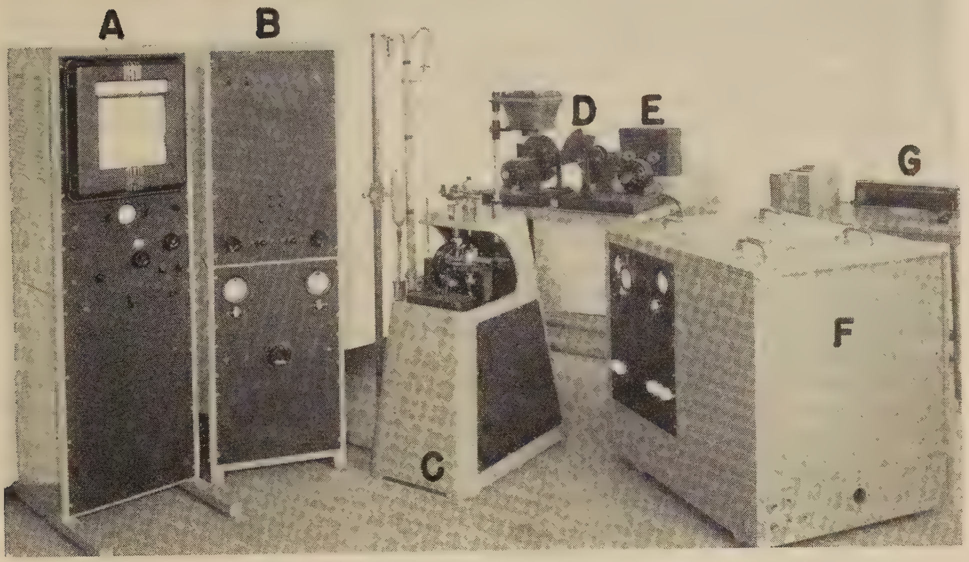

Fig. 1. The ultra-microspectrophotometer. For explanation see the text.

length ranges, for precision measurements of extremely high or low absorptions, for investigations with very low intensities of light, and for exact measurements of dichroism.

In the figure showing the apparatus, C is a heavy stand of cast iron and steel. It contains the source of light, the monochromator, the extremely stable carrier for interchangeable measuring tables for different kinds of specimens, as well as arrangements for moving the specimen during the measurements. It also houses the holder for the lenses, mechanically separated from the object carrier; the holder for the eyepiece; the mechanism for centering the points to be measured; the fluorescence eyepiece; and a microcamera. The highly magnified image is projected over the photocell-housing E. The shutters and diaphragm for the photocells are regulated from C. The racks A and B contain, respectively, the electronic equipment, and the controls for the different parts of the instrument, including the sources of light.

The instrument is designed both for point measurements (measurement of the detailed absorption spectrum of an extremely small, defined measuring area) and scanning measurements, in which the objéct is moved across a very narrow measuring beam. In the standard equipment, the maximum movement is 60 μ, with a reproducibility considerably better than 0.1 μ. (With special tables, any length of movement can be obtained.) In both types of measurement, the object is moved, whereas the whole optical arrangement remains stationary. This implies that full use can be made of the optics which—in the ultraviolet region—are often subject to residual errors. It also makes it easier to keep the incident light energy on a low level. For scanning measurements, the object is moved automatically, at the chosen rate, through the measuring ray in the axis of the optical system. The movement of the object is coupled electrically to the movement of the paper in the XY recorder for registering the light transmission.

The standard equipment can be used for a measuring surface down to 0.1 μ^2 (the limit of resolution of the high-power refraction lenses). In routine work, accuracy of more than 2 per cent can easily be obtained in the absorption range 10–90 per cent, if permitted by the nature of the material. The exact position of the point to be measured can be determined with an accuracy closely corresponding to the limit of resolution of the optics. The size of the measuring point (i.e., the small measuring area) can be regulated and checked exactly. The energy of the incident light is low, and the object is illuminated only during the actual readings.

The standard equipment is intended only for use with two photocells. RCA photomultipliers in dry-ice cooled housings are suitable both for point measurements and for scanning measurements. For the point measurements, an oscilloscope (which is easy to read at a distance) is used as a zero instrument. In the scanning measurements, the transmission is recorded. In both cases, a second photocell is used to compensate for the variations in the source of light, which are both considerable and irregular when discharge lamps are used. With variations in the lamp output up to 20 per cent, the errors in measurement are less than 2 per cent.

The lower part of the object table can be used for microspectrophotometry of photographic plates with movements up to 20 mm. For recording parts of the absorption spectrum of an individual point in the specimen, the object-table drive can be disconnected and switched to the wavelength drum.

The dimensions of the lens holder have been selected to suit both the refraction optics and the reflection optics of the types available on the market. A polarization prism can be inserted under the condenser for rough measurements of dichroism. The connection for the source of light is constructed to facilitate the interchange of different sources. It also permits easy replacement of the lamps; this is useful in view of the short lifetime of discharge lamps when used in ultraviolet.

In the middle ultraviolet region, the mercury high-pressure lamp is the most suitable source of light. This lamp can be used for measurements in a practically continuous spectrum down to 2570 Å, and in individual wavelength below this region. It is both easily obtained and cheap. The continuous spectrum of Siemens' high-pressure xenon lamp is a great advantage; this applies in particular to continuous direct recording of parts of the absorption spectrum and to measurements in very short wavelengths. Since it is still hard to obtain, it is, however, impossible to state whether it is economical in use. We made our tests with a Siemens lamp kindly placed at our disposal by Dr. E. Blout.

In the visible region, the usual sources of light can be used. For continuous recording of absorption spectra in the visible range—for instance, ultra-microcolorimetric work in very small cuvettes—the instrument can be used with an incandescent lamp as the source of light.

As stated earlier, various accessories can be fitted to the instrument, so that it can be used for the same purposes as the large universal spectrophotometer. Mention may be made of the arrangement with precision sectors (D in figure) with remote controls for highly accurate measurements of extremely high and low absorptions, respectively; a source of light for the 2700–2000 Å region (F in the figure), a high-energy rotating spark-gap fed with high frequency current. At these shorter wavelengths, the electronic equipment of the standard set is not particularly suitable. It is therefore replaced by an arrangement with a fluorescent plate and a single-multiplier system. In rack A, space is provided for this system, as well as for a logarithmic amplifier to permit recording of the extinction instead of the transmission. Among the other accessories are a special table for densitometry of X-ray ultra-microspectrographs and an arrangement that allows scanning of an object in a large number of parallel lines.

Complete equipment for a laboratory for optical microspectrophotometry also includes a set for ultraviolet microscopy, an arrangement for the standardization of the photographic material, and one for densitometry on the photographic plates. The instrument described may also be used for densitometry; a photoelectric microdensitometer is therefore superfluous. The rotating spark-gap (attachment F) is also an excellent source of light for ultraviolet microscopy, which may be performed in the photoelectric photometer if desired. The spark-gap is also suitable for use in photographic-photometric work.

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AUTHOR INDEX

A

Abercrombie, M., and Heaysman, J. E. M. Observations on the social behaviour of cells in tissue culture, 111
Åkerman, L. See Hammarsten, 404
Alfert, M., and Swift, H. Nuclear DNA constancy: A critical evaluation of some exceptions reported by Lison and Pasteels, 455
Allen, L. See Christiansen, 10
Anderson, N. G. Studies on isolated cell components. VI, 361
Åqvist, S. See Hammarsten, 404

B

Bahr, G. F. Gallocyanin — indiumalum, electron — stains I, 551
Bairati, A., and Lehmann, F. E. Structural and chemical properties of the plasmalemma of *Amoeba proteus*, 220
Barton, A. D. See Laird, 147
Bisset, K. A., and Hale, C. M. F. Complex cellular structure in bacteria, 449
Björklund, U. See Borei, 234
Bohus Jensen, A. The effect of trypsin on the cross fertilizability of sea urchin eggs, 325
Borei, H. G., and Björklund, U. Effect of versene on sea urchin eggs, 234
Brenner, S. The chromatic nuclear membrane, 257
Bucher, O., and Gattiker, R. Contribution à l'étude des cellules binucléées dans des cultures de fibroblastes, 461

C

Caspersson, T. *et al.*, A high resolution ultramicrospectrophotometer for large scale biological work, 560
Chambers, E. L., and Mende, T. Alterations of the inorganic phosphate and arginine phosphate content in sea urchin eggs following fertilization, 508
Chen, P. S. The rate of oxygen consumption in the lethal hybrid between *Triton* ♀ and *Salamandra* ♂, 275
Christiansen, G. S. *et al.* A culture chamber for the continuous biochemical and morphological study of living cells in tissue culture, 10
Christiansen, G. S. See Danes, 234
Cooperstein, S. J., and Lazarow, A. Studies on the mechanism of Janus Green B staining of mitochondria. III, 82
Cooperstein, S. J. *et al.* Studies on the mechanism of Janus Green B staining of mitochondria. II, 69
Cooperstein, S. J. See Lazarow, 56

D

Danes, B. *et al.* A Cartesian diver balance-capillary respirometer for metabolic studies of tissue cultures, 234
Danes, B. See Christiansen, 10
Danielsson, C. E. See Snellman, 436
Dass, C. M. S. See Seshachar, 248
Dianzani, M. U. Modification of some enzymatic characters of a strain of *Staphylococcus albus* obtained by treatment with desoxyribonucleic acid from other staphylococci, 311
Djurle, E., and Hallén, O. A double-slit interferometer for the determination of the thickness of the reference system used in X-ray microradiography, 301
Doré, C. F. See Lister, 542

E

Elfvin, L. G. The supravital staining with Janus Green B of mitochondria in the retinal rods of the guinea-pig eye, 554
Eliasson, N. See Hammarsten, 404

F

Fernandes, J. F., and Junqueira, L. C. U. Respiration, glycolysis and energy rich phosphorus compounds in secreting and non secreting rat submaxillary glands, 329
Finean, J. B. Further observations on the structure of myelin, 202
Finean, J. B. *et al.* Submicroscopic organisation of some layered lipoprotein structures (nerve myelin, retinal rods and chloroplasts), 557
Forssberg, A. The determination of small amounts of P^{32} labeled DNA in tissue by co-precipitation with an inactive carrier, 197
Fries, N. See von Hofsten, 530

G

Garber, B. Quantitative studies on the dependence of cell morphology and motility upon the fine structure of the medium in tissue culture, 132
Garvey, J. S. See Van Winkle, 38
Gattiker, R. See Bucher, 461
Golden, A. See Schaefer, 545
Gustafson, T. See Lenique, 400

H

Haddad, S. A. See Lindegren, 549
Hale, C. M. F. See Bisset, 449
Hallén, O. See Djurle, 301
Hammarsten, E. *et al.* Studies on the hemoglobin formation during regenerative erythropoiesis, 404
Hazeltine, P. See Ritchie, 261
Heaysman, J. E. M. See Abercrombie, 111
Hofsten, von A., See von Hofsten, 530
Hofsten, von, B. *et al.*, Continuous liquid culture of the fungus *Ophiostoma multiannulatum*, 530
Hörstadius, S. See Lenicque, 400

J

Jacobsson, F. See Caspersson 560
Junqueira, L. C. U. See Fernandes, 329

K

Kriszat, G. Die Wirkung von Perjodat auf den Zustand der Plasmamembran des Seeigeleis, 420

L

Laird, A. K. *et al.* Separation of mitochondria into two morphologically and biochemically distinct types, 147
Lazarow, A. See Cooperstein, 69, 82
Lazarow, A., and Cooperstein, S. J. Studies on the mechanism of Janus Green B staining of mitochondria. I, 56
Lehmann, F. E. See Bairati, 220
Leinfelder, P. J. See Christiansen, 10; Danes, 234
Lenicque, P. *et al.* Change of distribution of mitochondria in animal halves of sea urchin eggs by the action of micromeres, 400
Leyon, H. The structure of chloroplasts. II, 520
Lindahl, P. E. On a normally occurring reduction division in somatic cells of the sea urchin embryo, 416
Lindegren, C. E., and Haddad, S. A. The control of nuclear and cytoplasmic synthesis by the nucleo-cytoplasmic ratio in *Saccharomyces*, 549
Lister, W. C., and Doré, C. F. Roller tube tissue culture apparatus, 542
Lomakka, G. See Caspersson 560
Lowman, F. G. Electron microscope studies of silver salmon spermatozoa (*Oncorhynchus kisutch* (Walbaum)), 335

M

MacFarlane, E. W. E. Cytological conditions in root tip meristem after gross antagonism of phenylmercuric poisoning, 375
Mandels, G. R. Localization of carbohydrases at the surface of fungus spores by acid treatment, 48
Markert, C. L. Lethal and mutagenic effects on ultraviolet radiation on *Glomerella conidia*, 427
Marshak, A., and Marshak, C. Desoxyribonucleic acid in *Arbacia* eggs, 288
Marshak, C. See Marshak, 288
Matoltsy, A. G. A study of the medullary cells of the hair, 98
Mende, T. See Chambers, 508
Minganti, A. The action of proteolytic enzymes on the vitelline membrane of *Psammechinus miliaris* eggs, 492
Mitchison, J. M. Observations on ultracentrifuging of human red cell ghosts, 536
Morán Fernándes-, H. A diamond knife for ultrathin sectioning, 255
Motomura, I. Secretion of sperm agglutinin in the fertilized eggs of the sea urchins, 187
Mudd, S., and Winterscheid, L. C. A note concerning bacterial cell walls, mitochondria and nuclei, 251

N

Nygaard, O. See Laird, 147

P

Palik, E. S. See Van Winkle, 38
Patterson, J. W. See Cooperstein, 69
Perlmann, P. Soluble antigens in sea urchin gametes and developmental stages, 394
Pfeiffer, H. H. Messungen des Silber-Dichroismus an Amyloidkomplexen *in vitro*, 443
Pomerat, C. M. Rotating nuclei in tissue cultures of adult human nasal mucosa, 191
Prebus, A. F. See Van Winkle, 38
Preer, J. R., and Stark, P. Cytological observations on the cytoplasmic factor "kappa" in *Paramecium aurelia*, 478

R

Ravanti, K. See Teir, 500
Renoll, M. W. See Van Winkle, 38

RIBI, E. Electron microscopic investigation of the cell wall organization of wood, 161
RIS, H. See Laird, 147
RITCHIE, D., and HAZELTINE, P. Mitochondria in *Allomyces* under experimental conditions, 261

S

SÄFSTRÖM, R., See Caspersson, 560
SCHAEFER, H. J., and GOLDEN, A. Microalignment of alpha radioautographs by means of a contact replica of the tissue pattern printed with visible light, 545
SELBY, C. C. Electron micrographs of mitotic cells of the Ehrlich mouse ascites tumor in thin sections, 386
SESHACHAR, B. R., and DASS, C. M. S. Evidence for the conversion of desoxyribonucleic acid (DNA), to ribonucleic acid (RNA) in *Epistylis articulata* From, (Ciliata: Peritricha), 248
SJÖSTRAND, F. S. See Finean, 557
SLAUTTERBACK, D. B. Electron microscopic studies of small cytoplasmic particles (microsomes), 173
SNELLMANN, O., and DANIELSSON, C. E. An experimental study of the biosynthesis of the reserve globulins in pea seeds, 436
STARK, P. See Preer, 478
STEINMANN, E. See Finean, 557
STENRAM, U. The nucleolar size in the liver cell of rats fed high and non protein diets, 539
STOKELY, P. S. The synthesis of nicotinic acid by *Chilomonas paramecium*, 320
SVENSSON, G., See Caspersson, 560
SWIFT, H. See Alfert, 455

T

TEIR, H., and RAVANTI, K. Mitotic activity and growth factors in the liver of the white rat, 500
THORELL, B. See Hammarsten, 404
TIJN, VAN, B. See Wagtendonk, 1

W

WAGTENDONK, VAN, W. J., and van TIJN, B. Cross reaction of serotypes 51 A, 51 B, and 51 D of *Paramecium aurelia*, variety 4, 1
WEBB, M. The intracellular distribution of the deoxypentosenucleases of mammalian tissues, 16
WEBB, M. The preparation and properties of the deoxypentosenucleases of calf-thymus and mouse tissue, 27
WINKLE, VAN, Q., *et al.* Electron microscopy of isolated chromosomes, 38
WINTERSCHEID, L. C. See Mudd, 251

SUBJECT INDEX

A

Allomyces, mitochondria in, under experimental conditions, Ritchie and Hazeltine, 261
Amoeba, properties of plasmalemma of, Bairati and Lehmann, 220
Amyloid complexes, measurement of silver dichroism *in vitro*, Pfeiffer, 443
Animal halves of sea urchin eggs, distribution of mitochondria in, Lenicque *et al.*, 400
Antigens, soluble, in sea urchin gametes and developmental stages, Perlmann, 394
Arbacia eggs, desoxyribonucleic acid in, Marshak and Marshak, 288
Arginine phosphate, alterations of, in sea urchin eggs following fertilization, Chambers and Mende, 508
Ascites tumor, Ehrlich mouse, electron micrographs of mitotic cells, Selby, 386

B

Bacteria-complex cellular structure in, Bisset and Hale, 449; nuclei in, Mudd and Winterscheid, 251
Binucleate cells in cultures of fibroblasts, Bucher and Gattiker, 461

C

Carbohydrases at the surface of fungus spores, localization of, Mandels, 48
Cartesian diver for metabolic studies of tissue cultures, Danes *et al.*, 234
Cell wall — nuclei in, Mudd and Winterscheid, 251; organization of wood electron microscopy of, Ribi, 161
Chilomonas, synthesis of nicotinic acid in, Stokely, 320
Chloroplast — lipoprotein structures in, Finean 557; structure studied by means of electron microscopy, Leyon 520
Chromosomes, isolated, electron microscopy of, van Winkle *et al.*, 38
Co-precipitation in the determination of P^{32}-labeled desoxyribose nucleic acid, Forssberg, 197
Cross fertilizability of sea urchin, effect of trypsin, Bohus Jensen, 325
Cross reaction of serotypes in *Paramecium*, variety 4, van Wagtendonk and van Tijn, 1
Culture chamber for tissue culture, Christiansen, 10
Culture, liquid, of the fungus *Ophiostoma multiannulatum*, von Hofsten, 530

D

Deoxypentosenuclease — intracellular distribution of, Webb, 16; preparation and properties of, Webb, 27
Desoxyribonucleic acid — conversion of, to ribonucleic acid, Seshachar and Dass, 248; determination of small amounts of P^{32} in, Forssberg, 197; in *Arbacia* eggs, Marshak and Marshak, 288; nuclear constancy of, Alfert and Swift, 455; treatment of *Staphylococcus* by, Dianzani, 311
Dichroism (silver) in amyloid complexes, Pfeiffer, 443
Differentiation of the chloroplast structure, in *Vallota* and *Taraxacum*, Leyon, 520

E

Electron microscopy of — cell wall organization of wood, Ribi, 161; chloroplast structure, Leyon, 520; cytoplasmic particles, Slautterback, 173; isolated chromosomes, Van Winkle *et al.*, 38; microsomes, Slautterback, 173; mitotic cells of Ehrlich mouse ascites tumor, Selby, 386; silver salmon spermatozoa, Lowman, 335
Electron stains, Bahr, 551
Epistylis articulata, conversion of desoxyribonucleic acid to ribonucleic acid in, Seshachar and Dass, 248
Erythropoiesis, regenerative, hemoglobin formation in, Hammarsten *et al.*, 404

F

Fertilization — alterations of the inorganic phosphate and arginine phosphate content, Chambers and Mende, 508; membrane of the sea urchin egg, Mitchison, 536
Fibroblasts — binucleate cells in cultures of, Bucher and Gattiker, 461; chick heart, speed of movement of, Abercrombie and Heaysman, 111

G

Gallocyanin — indiumalum, BAHR, 551
Globulins, biosynthesis of reserve, in pea seeds, SNELLMAN and DANIELSSON, 436
Glomerella conidia, lethal and mutagenic effects of ultraviolet radiation on, MARKERT, 427
Glycolysis in submaxillary glands, FERNANDES and JUNQUEIRA, 329
Growth factors in the liver of the white rat, TEIR and RAVANTI, 500

H

Hair, medullary cells of, MATOLTSY, 98
Hemoglobin formation during regenerative erythropoiesis, HAMMARSTEN *et al.*, 404
Hybrid, lethal, between *Triton* ♀ and *Salamandra* ♂, oxygen consumption in, CHEN, 275

I

Interferometer, a double-slit, for the determination of thickness, DJURLE and HALLÉN, 301

J

Janus Green B — derivatives of; reactions and properties of, COOPERSTEIN *et al.*, 69; reduction of, by isolated enzyme systems, COOPERSTEIN and LAZAROW, 82; staining of mitochondria, LAZAROW and COOPERSTEIN, 56; staining of mitochondria in the retinal rods with, ELFVIN, 554

L

Leukaemic tissue, mouse, deoxypentosenucleases of, WEBB, 27
Lethal and mutagenic effects of ultraviolet radiation, on *Glomerella*, MARKERT, 427
Lipoprotein structures, organisation of, FINEAN 557
Liver cells — nucleolar size in, of rats fed high and non protein diets, STENRAM, 539

M

Medullary cells of hair, MATOLTSY, 98
Membrane, nuclear, BRENNER, 257
Meristem, in root tip, cytological conditions, after gross antagonism of phenylmercuric poisoning, MACFARLANE, 375
Micromeres, action of, on distribution of mitochondria in sea urchin eggs, LENICQUE *et al.*, 400
Microradiography, X-ray, determination of the thickness of the reference system, DJURLE and HALLÉN, 301
Microsomes, electron microscopic studies of, SLAUTTERBACK, 173
Microspectrophotometer, ultra, for large scale biological work, CASPERSSON, 560
Mitochondria — action of micromeres on distribution of sea urchin eggs, LENICQUE *et al.*, 400; bacterial, MUDD and WINTERSCHED, 251; in *Allomyces* under experimental conditions, RITCHIE and HAZELTINE, 261; in the retinal rods of the guinea-pig eye, staining with Janus Green B, ELFVIN, 554; mechanism of Janus Green B staining of, LAZAROW and COOPERSTEIN, 56; separation of, into two morphologically and biochemically distinct types, LAIRD *et al.*, 147
Mitosis — and growth factors in the liver of the white rat, TEIR and RAVANTI, 500; electron micrographs of, in thin sections, SELBY, 386
Myelin, structure of, FINEAN, 202

N

Nicotinic acid, synthesis of, by *Chilomonas paramecium*, STOKELY, 320
Nuclear membrane in rat liver cells, BRENNER, 257
Nucleases, effects on rat liver nuclei, ANDERSON, 361
Nuclei — bacterial, MUDD and WINTERSCHEID, 251; rat liver, effects of nucleases and proteases on, ANDERSON, 361
Nucleo-cytoplasmic ratio in *Saccharomyces*, LINDEGREN and HADDAD, 549

O

Oncorhynchus, spermatozoa of, LOWMAN, 335
Ophiostoma multiannulatum, liquid culture of, VON HOFSTEN, 530
Oxygen consumption in the lethal hybrid between *Triton* ♀ and *Salamandra* ♂, CHEN, 275

P

Paramecium, cytological observations on the cytoplasmic factor "kappa" in, PREER and STARK, 478
Particles, small cytoplasmic, electron microscopy of, SLAUTTERBACK, 173
Pea seeds, biosynthesis of the reserve globulins, SNELLMAN and DANIELSSON, 436
Periodate, action of, on the properties of the plasma membrane, KRISZAT, 420
Phenylmercuric poisoning in root tip meristem after gross antagonism, MACFARLANE, 375
Phosphate, inorganic, alterations of content in sea urchin eggs following fertilization, CHAMBERS and MENDE, 508
Phosphorus, energy rich, in submaxillary glands, FERNANDES and JUNQUEIRA, 329

Plasmalemma of *Amoeba*, properties of, Bairati and Lehmann, 220
Proteases, effects on rat liver nuclei, Anderson, 361

R

Radioautographs, alpha, micro-alignment of, Schaefer and Golden, 545
Reduction-division in somatic cells of the sea urchin embryo, Lindahl, 416
Respiration in submaxillary glands, Fernandes and Junqueira, 329
Retinal rods, lipoprotein structure in, Finean *et al.*, 557

S

Saccharomyces, control of nuclear and cytoplasmic synthesis, Lindegren and Haddad, 549
Sea urchin - action of periodate on the properties of the plasma membrane, Kriszat, 420; action of proteolytic enzymes of the vitelline membrane of *Psammechinus* eggs, Minganti, 492; change of distribution of mitochondria in eggs, Lenicque *et al.*, 400; desoxyribonucleic acid in *Arbacia* eggs, Marshak and Marshak, 288; effect of trypsin on cross fertilizability, Bohus Jensen, 325; effect of versene on eggs, Borei and Björklund, 216; fertilization membrane, thickness of, Mitchison, 536; inorganic phosphate and arginine phosphate content in, Chambers and Mende, 508; reduction-division in somatic cells of, Lindahl, 416; secretion of sperm agglutinin in the fertilized eggs of, Motomura, 187; soluble antigens in gametes and developmental stages, Perlmann, 394
Secretion in submaxillary glands, Fernandes and Junqueira, 329
Sectioning, ultrathin, a diamond knife for, Fernández-Morán, 255
Serotypes in *Paramecium*, van Wagtendonk and van Tijn, 1
Sperm agglutinin in the fertilized eggs of sea urchins, Motomura, 187
Spermatozoa, electron microscopy of, in silver salmon, Lowman, 335
Staphylococcus, modification of enzymatic characters of, Dianzani, 311
Submaxillary glands, respiration, glycolysis and energy rich phosphorus compounds in, Fernandes and Junqueira, 329

T

Taraxacum, differentiation of the chloroplast structure in, Leyon, 520
Thymus, deoxypentosenucleases of, Webb, 27
Tissue culture — a Cartesian diver balance-capillary respirometer for metabolic studies of, Danes *et al.*, 234; biochemical and morphological study of living cells in, Christiansen *et al.*, 10; dependence of cell morphology and motility upon structure of the medium, Garber, 132; of adult human nasal mucosa, rotating nuclei in, Pomerat, 191; roller tube apparatus for, Lister and Doré, 542; social behaviour of cells in, Abercrombie and Heaysman, 111
Trypsin, effect on cross fertilizability, Bohus Jensen, 325
Tumor, Ehrlich mouse, mitosis in, electron micrography of, Selby 386

U

Ultrathin sectioning, a diamond knife for, Fernández-Morán, 255
Ultraviolet radiation, lethal and mutagenic effects on *Glomerella conidia*, Markert, 427

V

Vallota, differentiation of the chloroplast structure in, Leyon, 520
Vitelline membrane, sea urchin egg, action of proteolytic enzymes on, Minganti, 492

W

Wood, electron microscopy of cell wall organization of, Ribi, 161

U. of Haw.